

IMS-130.0
4564/85124

IMMUNOGENIC HBc CHIMER PARTICLES STABILIZED
WITH AN N-TERMINAL CYSTEINE

CROSS-REFERENCE TO RELATED APPLICATION

This a continuation-in-part of application
Serial No. 09/930,915, filed August 15, 2001, whose
disclosures are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to the
intersection of the fields of immunology and protein
engineering, and particularly to a chimeric hepatitis
B virus (HBV) nucleocapsid protein that is engineered
for both enhanced stability of self-assembled
particles via an N-terminal cysteine residue and the
display of an immunogenic epitope.

BACKGROUND OF THE INVENTION

The family hepadnaviridae are enveloped
DNA-containing animal viruses that can cause
hepatitis B in humans (HBV). The hepadnavirus family
includes hepatitis B viruses of other mammals, e.g.,
woodchuck (WHV), and ground squirrel (GSHV), and
avian viruses found in ducks (DHV) and herons (HeHV).
Hepatitis B virus (HBV) used herein refers to a
member of the family hepadnaviridae that infects
mammals, as compared to a virus that infects an avian
host, unless the discussion refers to a specific
example of a non-mammalian virus.

The nucleocapsid or core of the mammalian
hepatitis B virus (HBV or hepadnavirus) contains a
sequence of 183 or 185 amino acid residues, depending

...the ... of ...

- 2 -

work with C-terminal-truncated proteins [Birnbaum et al., (1990) *J.Virol.* **64**, 3319-3330].

The hepatitis B nucleocapsid or viral core protein (HBc) has been disclosed as an immunogenic carrier moiety that stimulates the T cell response of an immunized host animal. See, for example, U.S. Patents No. 4,818,527, No 4,882,145 and No. 5,143,726. A particularly useful application of this carrier is its ability to present foreign or heterologous B cell epitopes at the site of the immunodominant loop that is present at about residue positions 70-90, and more usually recited as about positions 75 to 85 from the amino-terminus (N-terminus) of the protein. Clarke et al. (1991) F. Brown et al. eds., *Vaccines 91*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp.313-318.

During viral replication, HBV nucleocapsids associate with the viral RNA pre-genome, the viral reverse transcriptase (Pol), and the terminal protein (derived from Pol) to form replication competent cores. The association between the nucleocapsid and the viral RNA pre-genome is mediated by an arginine-rich domain at the carboxyl-terminus (C-terminus). When expressed in heterologous expression systems, such as *E.coli* where viral RNA pre-genome is absent, the protamine-like C-terminus; i.e., residues at positions 150 through 183, can bind *E.coli* RNA. Zhang et al. (1992) *JBC*, **267**(13) 9422-29.

HBcAg is a particulate protein derived from the hepatitis B virus that has been proposed as a carrier for heterologous epitopes. The relative immunogenicity of HBsAg (HBs) has been compared with HbcAg (HBc), and the ability of each to evoke immune responses in different genetic backgrounds [Milich et al., *Science*, (1986)

234(4782): p. 1398-1401]. These data emphasize the higher immunogenicity of HBc relative to HBs, and the universal responsiveness to HBc, irrespective of genetic background.

For example, HBc is more than 300 times more immunogenic than HBs in BALB/c mice; and, although both B10.S and B10.M mice are non-responders to HBs, every strain tested is responsive to HBc. These results re-emphasize the suitability of HBc as a vaccine carrier and specifically, its superiority over HBs, hence the selection of HBc as opposed to HBs to carry heterologous epitopes. These facets of HBc are thought to be important in influenza vaccine development, because they address issues of genetic restriction and inadequate antibody titers.

Another advantage of the HBc carrier is the fact that it does not require complex adjuvants for efficacy. This is due to the high inherent immunogenicity of the particle. A comparison of the immunogenicity of HBc-*P. berghei* particles showed that alum, which is approved for human use, was more effective than either IFA or CFA [Schodel et al., *J. Exp. Med.*, (1994) **180**(3): p. 1037-46]. The importance of this observation is highlighted by toxicity problems associated with newer, more complex adjuvants as was recently noted in clinical trials of SKB's candidate malaria vaccine [Stoute et al., *N. Engl. J. Med.*, [1997] **336**(2): p. 86-91].

In an application as a vaccine carrier moiety, it is preferable that the HBV nucleocapsids not bind nucleic acid derived from the host. Birnbaum et al. (1990) *J. Virol.*, **64**:3319-3330 showed that the protamine-like C-terminal domain of HBV nucleocapsids could be deleted without interfering with the protein's ability to assemble into virus-

like particles. It is thus reported that proteins truncated to about position 144; i.e., containing the HBc sequence from position one through about 144, can self-assemble, whereas deletions beyond residue 139 abrogate capsid assembly [F. Birnbaum & M. Nassal (1990) *J. Virol.*, 64: 3319-30] [Seifer et al., (1995) *Intervirology*, 38:47-62].

Zlotnick et al., (1997) *Proc. Natl. Acad. Sci., USA*, 94:9556-9561 studied the assembly of full length and truncated HBc proteins in to particles. In addition to discussing full length molecules, those authors reported the preparation of a truncated protein that contained the HBc sequence from position 1 through 149 in which the cysteines at positions 48, 61 and 107 were each replaced by alanines and in which a cysteine residue was added at the C-terminus (position 150). That C-terminal mercaptan was used for linkage to a gold atom cluster for labeling in electron microscopy.

More recently, Metzger et al. (1998) *J. Gen. Virol.*, 79:587-590 reported that the proline at position 138 (Pro-138 or P138) of the human viral sequence is required for particle formation. Those authors also reported that assembly capability of particles truncated at the carboxy-terminus to lengths of 142 and 140 residues was affected, with assembly capability being completely lost with truncations resulting in lengths of 139 and 137 residues.

Several groups have shown that truncated particles exhibit reduced stability relative to standard hepatitis B core particles [Galena et al. (1989) *J. Virol.*, 63:4645-4652; Inada, et al. (1989) *Virus Res.*, 14:27-48], evident by variability in

Such HBc chimeras often appear to have a less ordered structure, when analyzed by electron microscopy, compared to particles that lack heterologous epitopes [Schodel et al., (1994) *J.Exp.Med.*, **180**:1037-1046]. In some cases the insertion of heterologous epitopes into C-terminally truncated HBc particles has such a dramatic destabilizing affect that hybrid particles cannot be recovered following heterologous expression [Schodel et al. (1994) *Infect. Immunol.*, **62**:1669-1676]. Thus,

many chimeric HBc particles are so unstable that they fall apart during purification to such an extent that they are unrecoverable or they show very poor stability characteristics, making them problematic for vaccine development.

The above Pumpens et al. (1995) *Intervirology*, 38:63-74 report lists particle-forming chimers in which the inserted polypeptide sequence is at the N-terminus, the C-terminus and between the termini. Insert lengths reported in that article are 24 to 50 residues at the N-terminus, 7 to 43 residues internally, and 11 to 741 residues at the C-terminus.

Kratz et al., (1999) *Proc. Natl. Acad. Sci., U.S.A.*, 96:1915-1920 recently described the *E. coli* expression of chimeric HBc particles comprised of a truncated HBc sequence internally fused to the 238-residue green fluorescent protein (GFP). This chimera contained the inserted GFP sequence flanked by a pair of glycine-rich flexible linker arms replacing amino acid residues 79 and 80 of HBc. Those particles were said to effectively elicit antibodies against native GFP in rabbits as host animals.

U.S. Patent No. 5,990,085 describes two fusion proteins formed from an antigenic bovine inhibin peptide fused into (i) the immunogenic loop between residues 78 and 79 and (ii) after residue 144 of carboxy-terminal truncated HBc. Expressed fusion proteins were said to induce the production of anti-inhibin antibodies when administered in a host animal. The titers thirty days after immunization reported in that patent are relatively low, being 1:3000-15,000 for the fusion protein with the loop insertion and 1:100-125 for the insertion after residue 144.

U.S. Patent No. 6,231,864 teaches the preparation and use of a strategically modified hepatitis B core protein that is linked to a hapten. The modified core protein contains an insert of one to about 40 residues in length that contains a chemically-reactive amino acid residue to which the hapten is pendentlly linked.

Recently published WO 01/27281 teaches that the immune response to HBc can be changed from a Th1 response to a Th2 response by the presence or absence, respectively, of the C-terminal cysteine-containing sequence of the native molecule. That disclosure also opines that disulfide formation by C-terminal cysteines could help to stabilize the particles. The presence of several residues the native HBc sequence immediately upstream of the C-terminal cysteine was said to be preferred, but not required. One such alternative that might be used to replace a truncated C-terminal HBc sequence was said to include a C-terminal cysteine and an optional sequence that defines an epitope from other than HBc.

Published PCT application WO 01/98333 teaches the deletion of one or more of the four arginine repeats present at the C-terminus of native HBc, while maintaining the C-terminal cysteine residue. That application also teaches that the deleted region can be replaced by an epitope from a protein other than HBc so that the HBc portion of the molecule so formed acts as a carrier for the added epitope.

Published PCT applications corresponding to PCT/US01/25625 and PCT/US01/41759 of the present inventor teach that stabilization of C-terminally truncated HBc particles can be achieved through the use of one or more

added cysteine residues in the chimer proteins from which the particles are assembled. Those added cysteine residues are taught to be at or near the C-terminus of the chimeric protein.

A structural feature whereby the stability of full-length HBc particles could be retained, while abrogating the nucleic acid binding ability of full-length HBc particles, would be highly beneficial in vaccine development using the hepadnaviral nucleocapsid delivery system. Indeed, Ulrich et al. in their recent review of the use of HBc chimers as carriers for foreign epitopes [Adv. Virus Res., 50: 141-182 (1998) Academic Press] note three potential problems to be solved for use of those chimers in human vaccines. A first potential problem is the inadvertent transfer of nucleic acids in a chimer vaccine to an immunized host. A second potential problem is interference from preexisting immunity to HBc. A third possible problem relates to the requirement of reproducible preparation of intact chimer particles that can also withstand long-term storage.

The above four published PCT applications appear to contain teachings that can be used to overcome the potential problems disclosed by Ulrich et al. As disclosed hereinafter, the present invention provides another HBC chimera that provides unexpectedly high titers of antibodies against influenza, and in one aspect also provides a solution to the problems of HBC chimera stability as well as the substantial absence of nucleic acid binding ability of the construct. In addition, a contemplated recombinant chimera exhibits minimal, if

[illegible]

117

117

117

reported that in wild type HBeAg, the cysteine residue at position -7 of the pre-core sequence, which is present when the core gene is translated from an upstream initiator methionine at position -30, is responsible for preventing particle formation and therefore facilitating the transition from particulate HBcAg to secreted, non-particulate HBeAg.

Based upon the above three publications, one would expect the inclusion of one or more cysteine residues at a position prior to that of the initiator methionine of HBc; i.e., at a residue position of less than one relative to the N-terminus of the sequence of SEQ ID NO:1, to actually destabilize hybrid particles rather than stabilize them. As will be seen from the discussion that follows, the present invention provides results that are contrary to those expectations.

BRIEF SUMMARY OF THE INVENTION

The present invention contemplates a recombinant hepadnavirus nucleocapsid protein; i.e., a hepatitis B core (HBc) chimeric protein [or chimer hepatitis B core protein molecule or HBc chimer molecule or just chimer] that self-assembles into particles after expression in a host cell. A recombinant chimer hepatitis B core (HBc) protein molecule up to about 515 amino acid residues in length is contemplated. That chimer molecule

(a) contains an HBc sequence of at least about 125 of the N-terminal 150 amino acid residues of the HBc molecule that includes (i) the HBc sequence of residue positions 5 through about 75 and about 85 through about 140, (ii) a peptide-bonded heterologous immunogenic epitope at one or more of

the N-terminus, in the HBc immunodominant loop or the C-terminus of the chimer, or (iii) a heterologous linker residue for a conjugated epitope present in the HBc immunodominant loop,

(b) contains one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence and zero to about three cysteine residues toward the C-terminus of the molecule from the C-terminal residue of the HBc sequence and within about 30 residues from the C-terminus of the chimer molecule [C-terminal cysteine residue(s)].

That chimer molecule (i) contains no more than about 20 percent conservatively substituted amino acid residues in the HBc sequence, (ii) self-assembles into particles that are substantially free of binding to nucleic acids on expression in a host cell. The particles are more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) and (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue.

A preferred recombinant hepatitis B virus core (HBc) protein chimer molecule has a length of about 135 to about 515 amino acid residues that contains four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV.

$$\frac{1}{n} \begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix} \begin{bmatrix} 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \end{bmatrix} = \frac{1}{n} \begin{bmatrix} 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \\ 10 \end{bmatrix} = \begin{bmatrix} 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \end{bmatrix} = \mathbf{1}_n$$

1911 (Published by the U.S. Government Printing Office, Washington, D.C.)

Chimer Domain III is an Hbc sequence from

Chimer molecule Domain IV comprises (i)

immunogenic sequence heterologous to HBc from position 150 to the C-terminus.

The chimer molecules (i) have an amino acid residue sequence in which no more than about 10 percent of the amino acid residues are substituted in the HBc sequence of the chimer and (ii) self-assemble into particles on expression in a host cell. The particles are substantially free of binding to nucleic acids and are more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) and (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue.

It is preferred that the HBc sequence of Domain I include the residues of position 5 through position 75 along plus at least an N-terminal cysteine residue. It is further preferred that a contemplated immunogen contain not only an N-terminal cysteine residue, but also contain one cysteine residue within Domain IV as noted above that is alone or in an amino acid residue sequence heterologous to that of HBc from position 150 to the C-terminus.

The before-mentioned self-assembled chimer molecule particles are a particularly contemplated embodiment of this invention. Another embodiment comprises an inoculum or vaccine that comprises an above HBc chimer particle that is dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When administered in an immunogenic effective amount to an animal such as a mammal or bird, an inoculum induces antibodies that immunoreact specifically with the

A particular benefit of the invention is that its use as a vaccine provides extraordinary antibody titers against pathogens such as influenza A.

Another benefit of the invention is that the recombinant immunogen is prepared easily and using well-known cell culture techniques.

Another advantage of the invention is that the immunogen is easily prepared using well-known recombinant techniques.

Yet another benefit of the invention is that a preferred immunogen exhibits greater stability on preparation than do other HBc chimeras.

Yet another advantage of the invention is that a contemplated immunogen is substantially free of nucleic acids.

Still further benefits and advantages will be apparent to the worker of ordinary skill from the disclosure that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings forming a portion of this disclosure

Fig.1, shown in two panels as Fig. 1A and Fig. 1B, provides an alignment of six published sequences for mammalian HBc proteins from six viruses. The first (SEQ ID NO:1), human viral sequence is of the ayw subtype and was published in Galibert et al. (1983) *Nature*, **281**:646-650; the second human viral sequence (SEQ ID NO:2), of the adw subtype, was published by Ono et al. (1983) *Nucleic Acids Res.*, **11**(6): 1747-1757; the third human viral sequence (SEQ ID NO:3), is of the adw2 subtype and was published by Valenzuela et al., Animal Virus Genetics, Field et al. eds., Academic Press, New York (1980) pages 57-70; the fourth human viral sequence (SEQ ID NO:4), is of the adyw subtype that was published by Pasek et al. (1979) *Nature*, **282**:575-579; the fifth sequence (SEQ ID NO:5), is that of the woodchuck virus that was published by Galibert et al. (1982) *J. Virol.*, **41**:51-65; and the sixth mammalian sequence, (SEQ ID NO:6), is that of the ground squirrel that was published by Seeger et al. (1984) *J. Virol.*, **51**:367-375.

Fig. 2 shows the modifications made to commercial plasmid vector pKK223-3 in the preparation of plasmid vector pKK223-3N used herein for preparation of recombinant HBc chimers. The modified sequence (SEQ ID NO:7) is shown below the sequence of the commercially available vector (SEQ ID NO:8). The bases of the added NcoI site are shown in lower case letters and the added bases are shown with double underlines, whereas the deleted bases are shown as dashes. The two restriction sites present in this segment of the sequence (NcoI and HindIII) are indicated.

Fig. 3 is an analytical size exclusion chromatography elution profile for ICC-1603 particles in which absorbance at 280 nm is shown on the ordinate and time in seconds is shown on the abscissa.

Fig. 4 is an analytical size exclusion chromatography elution profile for ICC-1590 particles as discussed for Fig. 3.

Fig. 5 is an analytical size exclusion chromatography elution profile for ICC-1560 particles as discussed for Fig. 3.

Fig. 6 is an analytical size exclusion chromatography elution profile for ICC-1605 particles as discussed for Fig. 3.

Fig. 7 is an analytical size exclusion chromatography elution profile for ICC-1604 particles as discussed for Fig. 3.

Fig. 8 is an analytical size exclusion chromatography elution profile for ICC-1438 particles as discussed for Fig. 3.

Fig. 9 is an analytical size exclusion chromatography elution profile for ICC-1492 particles as discussed for Fig. 3.

Fig 10 is a photograph of an SDS-PAGE analysis under reducing conditions following particle preparation that shows the ICC-1438 monomer construct was unstable after aging (Lane 2) as compared to the ICC-1492 construct (Lane 3), with HBC-149 (Lane 1), ICC-1475 (Lane 4) and ICC-1473 (Lane 5) serving as additional molecular weight controls.

Fig. 11, taken from PCT/US01/25625 (ICC-102.2) illustrates a reaction scheme (Scheme 1) that shows two reaction sequences for (I) forming an activated carrier for pendently linking a hapten to a

chimeric hepatitis B core protein (sm-HBc) particle using sulpho-succinimidyl 4-(N-maleimidomethyl)-cyclohexane 1-carboxylate (sulpho-SMCC), and then (II) linking a sulfhydryl-terminated (cysteine-terminated) hapten to the activated carrier to form a conjugate particle. The sm-HBc particle is depicted as a box having a single pendent amino group (for purposes of clarity of the figure), whereas the sulfhydryl-terminated hapten is depicted as a line terminated with an SH group.

Definitions

Numerals utilized in conjunction with HBc chimers indicate the position in the HBc ayw amino acid residue sequence of SEQ ID NO:1 at which one or more residues has been added to or deleted from the sequence, regardless of whether additions or deletions to the amino acid residue sequence are present. Thus, HBc149 indicates that the chimera ends at residue 149, whereas HBc149 + C150 indicates that that same chimera contains a cysteine residue at HBc position 150 relative to the sequence numbers of SEQ ID NO:1.

The term "antibody" refers to a molecule that is a member of a family of glycosylated proteins called immunoglobulins, which can specifically bind to an antigen.

The word "antigen" has been used historically to designate an entity that is bound by an antibody or receptor, and also to designate the entity that induces the production of the antibody. More current usage limits the meaning of antigen to that entity bound by an antibody or receptor, whereas the word "immunogen" is used for the entity that

[illegible]

reported by Galibert et al., (1979) *Nature*, 281:646-650 (SEQ ID NO:1). The polypeptide portions of at least chimer Domains I, II and III are believed to exist in a similar tertiary form to the corresponding sequences of naturally occurring HBcAg.

As used herein, the term "fusion protein" designates a polypeptide that contains at least two amino acid residue sequences not normally found linked together in nature that are operatively linked together end-to-end (head-to-tail) by a peptide bond between their respective carboxy- and amino-terminal amino acid residues. The fusion proteins of the present invention are HBc chimer molecules that induce the production of antibodies that immunoreact with a polypeptide that corresponds in amino acid residue sequence to the polypeptide portion of the fusion protein.

The phrase "hepatitis B" as used here refers in its broadest context to any member of the family of mammalian hepadnaviridae, as discussed before.

The words "polypeptide" and "peptide" are used interchangeably throughout the specification and designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acids. Polypeptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms that are salts. It is well understood in the art that amino acid residue sequences contain acidic and basic groups, and that the particular ionization state exhibited by the peptide is dependent on the pH value of the surrounding medium when the peptide is in solution, or that of the medium from which it was

obtained if the peptide is in solid form. Thus, "polypeptide" or its equivalent terms is intended to include the appropriate amino acid residue sequence referenced. A peptide or polypeptide is always shown herein from left to right and in the direction from amino-terminus (N-terminus) to carboxy-terminus (C-terminus).

The term "residue" is used interchangeably with the phrase amino acid residue. All amino acid residues identified herein are in the natural or L-configuration. In keeping with standard polypeptide nomenclature, [*J. Biol. Chem.*, 243, 3557-59 (1969)], abbreviations for amino acid residues are as shown in the following Table of Correspondence.

TABLE OF CORRESPONDENCE		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
Z	Glx	L-glutamic acid

W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
B	Asx	L-aspartic acid

or

C	Cys	L-cysteine
---	-----	------------

The present invention contemplates a chimeric hepadnavirus nucleocapsid protein; i.e., a recombinant hepatitis B core (HBc) protein, that is engineered to (a) display an immunogenic B cell or T cell epitope, or a linker for attachment of an immunogenic B cell or T cell epitope, (b) exhibit enhanced stability on formation when present in a self-assembled particle, as well as exhibit (c) a substantial absence of nucleic acid binding as a self-assembled particle. A contemplated HBc chimera is truncated at the C-terminus of the molecule relative to a native HBc molecule.

- 22 -

lysine residues downstream of (toward the carboxy-terminus from) HBc residue position 149 so that the self-assembled particles are substantially free of nucleic acid binding.

For ease of discussion, contemplated chimer sequences and sequence position numbers referred to herein are based on the sequence and position numbering of the human hepatitis B core protein of subtype ayw [Galibert et al., (1979) *Nature*, **281**:646-650] that is shown in SEQ ID NO:1. It is to be understood, however, that in view of the great similarity between the mammalian hepadnavirus capsid protein sequences and similar particle formation exhibited by those proteins, which are well-known to skilled workers, a discussion regarding human HBc subtype ayw is also applicable to subtype adw, as well as the woodchuck and ground squirrel proteins. As a consequence of those great similarities, HBc sequences are recited generally herein as a "HBc" sequence, unless otherwise stated.

In one embodiment, a contemplated HBc chimera is up to about 515 residues in length and contains

(a) an HBc sequence of at least about 125 of the N-terminal 150 amino acid residues of the HBc molecule that includes (i) the HBc sequence of residue positions 5 through about 75 and about 85 through about 140, (ii) a peptide-bonded heterologous immunogenic epitope at one or more of the N-terminus, in the HBc immunodominant loop or the C-terminus of the chimera, or (iii) a heterologous linker residue for a conjugated epitope present in the HBc immunodominant loop, and

(b) one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1 from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence and zero to about three cysteine residues toward the C-terminus of the molecule from the C-terminal residue of the HBc sequence and within about 30 residues from the C-terminus of the chimer molecule [C-terminal cysteine residue(s)].

That chimer molecule (i) contains no more than about 20 percent conservatively substituted amino acid residues in the HBc sequence, (ii) self-assembles into particles that are substantially free of binding to nucleic acids on expression in a host cell. The particles are more stable on formation than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) and (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue.

A contemplated chimer molecule contains a cysteine residue at a position of about -20 to about +1 relative to the N-terminus of HBc as is illustrated in Fig. 1 and SEQ ID NO:1. The concept of a negative amino acid position is usually associated with a leader sequence such as the precore sequence of HBc. That concept is used similarly here in that one can simply align a given chimer molecule sequence with that of SEQ ID NO:1 to determine the position of the chimer that corresponds to that of

the starting methionine residue of position +1 of HBc. Inasmuch as amino acid residue sequences are normally shown from left to right and in the direction from N-terminus to C-terminus, any aligned chimer molecule residue to the left of the position occupied by the HBc start methionine has a negative position. A contemplated cysteine residue can occur at a position about twenty residues to the left of the aligned start methionine of HBc to the position corresponding to that start methionine.

In one aspect, a preferred HBc chimer has a sequence of about 135 to about 515 L- α -amino acid residues and contains four serially peptide-linked domains; i.e., Domains I, II, III and IV. Those four domains are linked together in the same manner as are native proteins; i.e., they are peptide-bonded to each other, as compared to polypeptides that contain residues of other than α -amino acids and therefore cannot form peptide bonds, those that contain D-amino acid residues, or oligopeptide conjugates in which two or more polypeptides are operatively linked through an amino acid residue side chain. A contemplated chimeric HBc protein can therefore be prepared by expression using the usual methods of recombinant technology.

Domain I of that chimer molecule comprises about 71 to about 110 amino acid residues whose sequence includes (i) at least the sequence of the residues of position 5 through position 75 of HBc, (ii) one to three cysteine residues at an amino acid position of the chimer molecule corresponding to amino acid position -20 to about +1, and preferably amino acid position -14 to about +1, from the N-terminus of the HBc sequence of SEQ ID NO:1 [N-

terminal cysteine residue(s)] in a sequence other than that of the HBc precore sequence, and (iii) an optional heterologous immunogenic epitope containing up to about 30 amino acid residues peptide-bonded to one of HBc residues 2-4. That heterologous immunogenic sequence, when present, is typically an epitope used to induce an immune response for a vaccine or inoculum.

Domain II of that chimer molecule comprises about 5 to about 250 amino acid residues peptide-bonded to HBc residue 75 of Domain I in which (i) zero to all residues in the sequence of HBc positions 76 to 85, and preferably at least four HBc residues, are present peptide-bonded to (ii) an optionally present sequence of one to about 245 amino acid residues that are heterologous to HBc and constitute a heterologous immunogenic epitope or a heterologous linker residue for a conjugated epitope. It is particularly preferred that the sequence of 10 residues of positions 76 through 85 (position 76-85 sequence) be present, but interrupted by one to about 245 residues of the heterologous linker or heterologous epitope.

Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85 of Domain II.

Chimer molecule Domain IV comprises (i) five through fourteen residues of an HBc amino acid residue sequence from position 136 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues [C-terminal cysteine residue(s)] within about 30 residues from the C-terminus of the chimer molecule, and (iii) zero to about 100 amino acid residues in an

immunogenic sequence heterologous to HBc from position 150 to the C-terminus. Preferably, Domain IV contains a sequence of zero to about 50 amino acid residues in a sequence heterologous to HBc, and more preferably that sequence is zero to about 25 residues. Domain IV also preferably contains one C-terminal cysteine residue.

The chimer molecules (i) have an amino acid residue sequence in which no more than about 10 percent of the amino acid residues are substituted in the HBc sequence of the chimer and (ii) self-assemble into particles on expression in a host cell. The particles are substantially free of binding to nucleic acids and are more stable than are particles formed from otherwise identical HBc chimer molecules that are free of any above-mentioned C-terminal cysteine residue(s) and (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue.

In one aspect, a contemplated chimer molecule contains a heterologous epitope at the N-terminus peptide-bonded to one of HBc residues 2-5. In another aspect, a contemplated chimer molecule contains a heterologous epitope or a heterologous linker residue for an epitope peptide-bonded near the middle of the molecule located between HBc residues 76 and 85 in the immunodominant loop. In a further aspect, a heterologous epitope is located at the C-terminal portion of the chimer molecule peptide-bonded to one of HBc residues 136-149. In yet other aspects, two or three heterologous epitopes are present at the above locations, or one or two heterologous epitopes are present along with a

$\frac{1}{2}, \frac{1}{3}, \frac{1}{4}, \frac{1}{5}, \frac{1}{6}, \frac{1}{7}, \frac{1}{8}, \frac{1}{9}, \frac{1}{10}, \frac{1}{11}, \frac{1}{12}, \frac{1}{13}, \frac{1}{14}, \frac{1}{15}, \frac{1}{16}, \frac{1}{17}, \frac{1}{18}, \frac{1}{19}, \frac{1}{20}, \frac{1}{21}, \frac{1}{22}, \frac{1}{23}, \frac{1}{24}, \frac{1}{25}, \frac{1}{26}, \frac{1}{27}, \frac{1}{28}, \frac{1}{29}, \frac{1}{30}, \frac{1}{31}, \frac{1}{32}, \frac{1}{33}, \frac{1}{34}, \frac{1}{35}, \frac{1}{36}, \frac{1}{37}, \frac{1}{38}, \frac{1}{39}, \frac{1}{40}, \frac{1}{41}, \frac{1}{42}, \frac{1}{43}, \frac{1}{44}, \frac{1}{45}, \frac{1}{46}, \frac{1}{47}, \frac{1}{48}, \frac{1}{49}, \frac{1}{50}, \frac{1}{51}, \frac{1}{52}, \frac{1}{53}, \frac{1}{54}, \frac{1}{55}, \frac{1}{56}, \frac{1}{57}, \frac{1}{58}, \frac{1}{59}, \frac{1}{60}, \frac{1}{61}, \frac{1}{62}, \frac{1}{63}, \frac{1}{64}, \frac{1}{65}, \frac{1}{66}, \frac{1}{67}, \frac{1}{68}, \frac{1}{69}, \frac{1}{70}, \frac{1}{71}, \frac{1}{72}, \frac{1}{73}, \frac{1}{74}, \frac{1}{75}, \frac{1}{76}, \frac{1}{77}, \frac{1}{78}, \frac{1}{79}, \frac{1}{80}, \frac{1}{81}, \frac{1}{82}, \frac{1}{83}, \frac{1}{84}, \frac{1}{85}, \frac{1}{86}, \frac{1}{87}, \frac{1}{88}, \frac{1}{89}, \frac{1}{90}, \frac{1}{91}, \frac{1}{92}, \frac{1}{93}, \frac{1}{94}, \frac{1}{95}, \frac{1}{96}, \frac{1}{97}, \frac{1}{98}, \frac{1}{99}, \frac{1}{100}$

[illegible]

The heterologous immunogenic epitope that

Domain III contains HBc residues 86 through

CONFIDENTIAL

Domain IV contains a sequence of at least five residues that are comprised of (i) a sequence of the residues of HBc positions 136 through 140, and preferably through 149, peptide-bonded to residue 135, (ii) zero to three cysteines residues and (iii) optionally can contain a sequence of a heterologous epitope of up to about 100 residues, particularly when the HBc sequence ends at residue 140, although a shorter sequence of up to about 25 residues is more preferred. That Domain IV heterologous sequence is heterologous to the sequence of HBc and is other than a sequence of HBc from position 150 to the HBc C-terminus. The heterologous sequence, when present in Domain IV, is preferably a T cell epitope, but can also be a B cell epitope as are usually present in one or the other of Domains I and II.

Domain IV can also contain zero to three cysteine residues and those Cys residues are present within about 30 residues of the carboxy-terminus (C-terminus) of the chimera molecule. Preferably, one cysteine (Cys) residue is present, and that Cys is preferably present as the carboxy-terminal (C-terminal) residue, unless a T cell epitope is present as part of Domain IV. When such a T cell epitope is present, the preferred Cys is preferably within the C-terminal last five residues of the HBc chimera.

In one embodiment, a particularly preferred chimera contains two heterologous epitopes. Those two heterologous epitopes are present in Domains I and II, or II and IV, or I and IV. One of the two heterologous epitopes is preferably a B cell epitope in some embodiments. In other embodiments, one of the two heterologous epitopes is a T cell epitope. More preferably, one of the two heterologous epitopes

is a B cell epitope and the other is a T cell epitope. In addition, a plurality of B cell epitopes can be present at the B cell epitope location and a plurality of T cell epitopes can be present at the T cell epitope location.

In the embodiments in which the chimer molecule contains a heterologous epitope in Domain II, it is preferred that that the sequence contain one or more B cell epitopes, that the HBc sequence between amino acid residues 76 and 85 be present, but interrupted by the heterologous epitope(s), and that the chimer further include one or more T cell epitopes in Domain IV peptide-bonded to one of HBc residues 140-149.

This same preference holds for those chimer molecules in which the heterologous linker residue for a conjugated epitope is present in Domain II, thereby providing one or more heterologous epitopes in Domain II, with residues 76 and 85 present, but interrupted by the heterologous linker residue, with a T cell epitope being present peptide-bonded to one of HBc residues 140-149. The particles formed from such chimer molecules typically contain a ratio of conjugated epitope to C-terminal peptide-bonded T cell epitope of about 1:4 to 1:1, with a ratio of about 1:2 being common.

In an illustrative structure of an above-described chimer molecule, a heterologous linker residue for a conjugated epitope is present in Domain II and a T cell epitope is present in Domain IV, with no additional B cell epitope being present in Domain II. Such a chimer exhibits immunogenicity of the T cell epitope, while exhibiting minimal, if any, HBc antigenicity as measured by binding of anti-loop

monoclonal antibodies in an ELISA assay as discussed hereinafter.

A preferred contemplated HBc chimer molecule contains a sequence of about 135 to about 515 residues. A preferred HBc chimer molecule containing two heterologous epitopes of preferred lengths of about 15 to about 50 residues each and a preferred HBc portion length of about 140 to about 149 residues has a sequence length of about 170 to about 250 amino acid residues. Particularly preferred chimer molecules containing two heterologous epitopes have a length of about 180 to about 210 residues. It is to be understood that a wide range of chimer molecule lengths is contemplated in view of the variations in length of the N- and C-terminal HBc portions and differing lengths of the several contemplated epitopes that can be inserted in the immunogenic loop.

A contemplated recombinant protein, after expression in a host cell, self-assembles to form particles that are substantially free of binding to nucleic acids. The contemplated HBc chimer particles are generally spherical in shape and are usually homogeneous in size for a given preparation. These chimeric particles thus resemble native HBc particles that have a similar shape and size and can be recovered from infected persons.

A contemplated chimer particle comprises previously discussed chimer molecules. More broadly, such a chimer particle comprises a chimeric C-terminal truncated HBc protein that has a sequence of at least about 125 of the N-terminal 150 residues and contains (i) a heterologous epitope peptide-bonded to one or more of the N-terminus, C-terminus or the

immunodominant loop, or a heterologous linker residue for an epitope in the immunodominant loop, and (ii) one to three N-terminal cysteine residues and zero to three C-terminal cysteine residues as previously described, and at least a 5 HBc residue sequence from position 135.

A contemplated particle is sufficiently free of arginine and/or lysine residues in Domain IV so that the self-assembled particles are substantially free of nucleic acid binding and exhibits a 280/260 absorbance ratio of about 1.2 to about 1.7, as discussed herein after. Thus, a contemplated chimeric protein is free of the HBc sequence between positions 150 and 183.

The presence of the above-discussed N-terminal cysteine residue(s) provides an unexpected enhancement of the ability of the chimer molecules to form stable immunogenic particles (discussed hereinafter). Thus, a contemplated HBc chimer particle immunogen tends to form particles that stay together upon collection and initial purification as measured by analytical size exclusion chromatography, whose details are discussed hereinafter.

Contemplated particles are more stable upon formation than are particles formed from otherwise identical HBc chimer molecules that (i) lack the N-terminal cysteine residue(s) or (ii) in which an N-terminal cysteine residue(s) present in a contemplated chimer molecule is(are) replaced by another residue and are also free of any above-mentioned C-terminal cysteine residue(s). In some instances, particles do not form unless an N-terminal cysteine is present. Examples of enhanced

stabilities for both types of sequences are illustrated in the Examples that follow.

A contemplated particle containing a N-terminal cysteine residue is also typically prepared in greater yield than is a particle assembled from a chimer molecule lacking a N-terminal cysteine. This increase in yield can be seen from the mass of particles obtained or from analytical gel filtration analysis using Superose® 6 HR as discussed hereinafter.

The substantial freedom of nucleic acid binding exhibited by contemplated particles can be readily determined by a comparison of the absorbance of the particles in aqueous solution measured at both 280 and 260 nm; i.e., a 280/260 absorbance ratio. The contemplated particles do not bind substantially to nucleic acids that are oligomeric and/or polymeric DNA and RNA species originally present in the cells of the organism used to express the protein. Such nucleic acids exhibit an absorbance at 260 nm and relatively less absorbance at 280 nm, whereas a protein such as a contemplated chimer absorbs relatively less at 260 nm and has a greater absorbance at 280 nm.

Thus, recombinantly expressed HBc particles or chimeric HBc particles that contain the arginine- and lysine-rich sequence at residue positions 150-183 (or 150-185) sometimes referred to in the art as the protamine region exhibit a ratio of absorbance at 280 nm to absorbance at 260 nm (280/260 absorbance ratio) of about 0.8. On the other hand, particles sufficiently free of arginine and lysine residues in Domain IV so that the self-assembled particles are substantially free of nucleic acid binding such as

particles that are free of the arginine-rich nucleic acid binding region of naturally occurring HBc like as those that contain fewer than three arginine or lysine residues or mixtures thereof adjacent to each other, or those having a native or chimeric sequence that ends at about HBc residue position 140 to position 149, exhibit a 280/260 absorbance ratio of about 1.2 to about 1.6. A more typical 280/260 absorbance ratio is about 1.4 to about 1.6. This range is due in large part to the number of aromatic amino acid residues present in Domains II and IV of a given chimeric HBc particle.

Domain I of a contemplated chimeric HBc protein constitutes an amino acid residue sequence of HBc beginning with at least amino acid residue position 5 through position 75, and Domain III constitutes a HBc sequence from position 86 through position 137. The sequences from any of the mammalian hepadnaviruses can be used for either of Domains I and III, and sequences from two or more viruses can be used in one chimera. Preferably, and for ease of construction, the human ayw sequence is used through out the chimera.

HBc chimeras having a Domain I that contains more than a deletion of the first three amino-terminal (N-terminal) residues have been reported to result in the complete disappearance of HBc chimera protein in *E. coli* cells. Pumpens et al., (1995) *Intervirology*, 38:63-74. On the other hand, a recent study in which an immunogenic 23-mer polypeptide from the influenza M2 protein was fused to the HBc N-terminal sequence reported that the resultant fusion protein formed particles when residues 1-4 of the native HBc sequence were replaced. Neirynck et al.

(October 1999) *Nature Med.*, 5(10):1157-1163. Thus, the art teaches that particles can form when an added amino acid sequence is present peptide-bonded to one of residues 2-4 of HBc, whereas particles do not form if no additional sequence is present and more than residues 1-3 are deleted from the N-terminus of HBc.

An N-terminal epitope sequence peptide-bonded to one of the first five N-terminal residues of HBc can contain a single cysteine residue or a sequence of up to about 30 residues that are heterologous to HBc. The one to three cysteine residues can be present at a convenient location in the sequence, but are typically near the C-terminus of the added sequence so that the added N-terminal cysteine residue(s) are at a position of about -20 to about +1, and more preferably at a position of about -14 to about +1, relative to the HBc N-terminus as shown in SEQ ID NO:1. Exemplary sequences include a B cell or T cell epitope such as those discussed and illustrated hereinafter (Tables A and B, respectively), the 23-mer polypeptide from the influenza M2 protein of Neirynck et al., above, that includes two cysteine residues, and variants of that sequence containing at least about 6 residues, a sequence of another (heterologous) protein such as β -galactosidase as can occur in fusion proteins as a result of the expression system used, or another hepatitis B-related sequence such as that from the Pre-S1 or Pre-S2 regions or the major HbsAg immunogenic sequence.

Domain II is a sequence of about 5 to about 250 amino acid residues. Of those residues, zero (none), and preferably at least 4 residues, and more preferably at least 8, constitute portions of the HBc

sequence at positions 76 to 85, and one to about 245 residues, and preferably one to about 50 residues are heterologous (foreign) to HBc. Thus, at least HBc residues 75 and 85 are present in Domains I and II, respectively. Those heterologous residues constitute (i) a heterologous linker residue for a epitope such as a B cell or T cell epitope or (ii) a heterologous B or T cell epitope that preferably contains 6 to about 50, more preferably about 15 to about 50, and most preferably about 20 to about 30 amino acid residues, and are positioned so that they are peptide-bonded between zero, or more preferably at least 4, to all of the residues of positions 76 to 85 of the HBc sequence. Heterologous B cell epitopes are preferably linked at this position by the linker residue or are peptide-bonded into the HBc sequence, and use of a B cell epitope is discussed illustratively hereinafter.

Those preferred at least 4 HBc residues can be all in one sequence such as residues 82-85, or can be split on either side of (flank) the heterologous residue(s) as where residues 76-77 and 84-85 are present or where residues 76 and 83-85 are present. More preferably, Domain II contains at least 8 residues of the HBc sequence from residue 76 to 85. Most preferably, the sequence of all 10 residues of positions 76 to 85 are present in the chimera.

The one to about 245 residues added to the HBc loop sequence is(are) heterologous to a HBc sequence. A single added heterologous residue is a heterologous linker residue for a B cell epitope as discussed before. The longer sequences, typically at least 6 amino acid residues long to about 50 amino acid residues long and more preferably about 15 to

about 50 residues in length, as noted before, are in a sequence that comprises a heterologous immunogen such as a B cell or T cell epitope, except for heterologous residues encoded by restriction sites.

Exemplary peptide B cell epitopes useful for both linkage to the linker residue after expression of a contemplated chimera and for expression within a HBc chimera at one or more of the N-terminus, within the immunogenic loop or at the C-terminus of the chimera are illustrated in Table A, below, along with the common name given to the gene from which the sequence is obtained, the literature or patent citation for published epitopes, and SEQ ID NO.

Table A

B Cell Epitopes

<u>Organism</u>	<u>Gene</u>	<u>Sequence</u>	<u>Citation*</u>	<u>SEQ ID NO</u>
<i>Streptococcus pneumoniae</i>	PspA1	KLEELSDKIDELDAE QKKYDEDQKKTEE-	1	9
	Psp2	KAALEKAASEEM- DKAVAAVQQA	1	10
<i>Cryptosporidium parvum</i>	P23	QDKPADAPAAEAPA- AEPAQQDKPADA	2	11
HIV	GP120	RKRIHIGPGR- AFYITKN	3	12
Foot-and-mouth virus	VP1	YNGECRYNRNA- VPNLRGDLQVL- AQKVARTLP	4	13

Influenza Virus
A8/PR8

HA

YRNLLWLTEK 8 14

Type A
(A8/PR8/34)

M2

SLLTEVETPIR-
NEWGCRCNGSSD 29 15
SLLTEVETPIR-
NEWGCRCNDSSD 29 16
SLLTEVETPIR-
NEWGARANDSSD 17
EQQSAVDADDS-
HFVSIELE 35 18
SLLTEVETPIR-
SLLTEVETPIR-
NEWGSRSDNDSSD 19
SLLTEVETPIR-
NEWGSRCDNDSSD 20
SLLTEVETPIR-
NEWGCRSDNDSSD 21
SLLTEVETPIR-
NEWGCRANDSSD 22
SLLTEVETPIR-
NEWGARCNDSSD 23
MSLLTEVETPIR-
NEWGCRCNDSSD 24
MSLLTEVETPIR-
NEWGSRSDNDSSD 25
MGISLLTEVETPIR-
NEWGCRCNDSSD-
ELLGWLWGI 26
MSLLTEVETPIR-
NEWGARANDSSD 27
MSLLTEVETPIR-
NEWGCRANDSSD 28
MSLLTEVETPIR-
NEWGARCNDSSD 29
MSLLTEVETPIR-
NEWGCRSDNDSSD 30
MSLLTEVETPIR-
NEWGSRCDNDSSD 31
X₁X₂X₃X₄X₅X₆X₇X₈-
TPIRNEX₁₅X₁₆X₁₇X₁₈X₁₉X₂₀-
X₂₁X₂₂X₂₃X₂₄ 32

Type B

NB

NNATFNVTNPNPISHIR 271

Yersinia
pestis

V Ag

DILKVIVDSMHH-
GDARSKLREELAE-
LTAEKLIYSVIQA-
EINKHLSSSGTIN-
IHDKSINLMDKNL-
YGYTDEEIFKASA-
EYKILEKMPQTTI-
QVDGSEKKIVSIK-
DFLGSENKRTGAL-
GNLKNSYSYNKDN-
NELSHFATTCSO 9 33

Haemophilus

influenza	pBOMP			
		CSSSNNDAA-		
		GNGAAQFGGY	10	34
		NKLGTVSYGEE		35
		NDEAAYSKN-		
		RRAVLAY		36
<i>Moraxella catarrhalis</i>	copB	LDIEKDKKK-		
		RTDEQLQAE-		
		LDDKYAGKGY	11	37
		LDIEKNKKK-		
		RTEAELQAE-		
		LDDKYAGKGY		38
		IDIEKKGKI-		
		RTEAELLAE-		
		LNKDYPGQGY		39
<i>Porphyromonas gingivalis</i>	HA	GVSPKVCKDVTV-		
		EGSNEFAPVQNL	12	40
		RIQSTWRQKTV-		
		DLPAGTKYV		41
<i>Trypanosoma cruzi</i>		KAAIAPAKAAA-		
		APAKAATAPA	14	42
<i>Plasmodium falciparum</i>	CS	(NANP) ₄	24	43
		NANPNVDP-		
		(NANP) ₃ NVDP		44
		NANPNVDP-		
		(NANP) ₃		45
		(NANP) ₃ NVDPNANP		46
		NANPNVDP-		
		(NANP) ₃ NVDPNANP		47
		NPNVDP (NANP) ₃ NV		48
		NPNVDP-		
		(NANP) ₃ NVDP		49
		NPNVDP (NANP) ₃ -		
		NVDPNA		50
		NVDP (NANP) ₃ NV		51
		NVDP (NANP) ₃ NVDP		52
		NVDP (NANP) ₃ -		
		NVDPNA		53
		DP (NANP) ₃ NV		54
		DP (NANP) ₃ NVDP		55
		DP (NANP) ₃ -		
		NVDPNA		56
<i>vivax</i>	CS	GDRADGQPAG-		
		DRADGQPAG	20	57

		RADDRAAGQP-		
		AGDGQPAG		58
		ANGAGNQPG-		
		ANGAGDQPG		59
		ANGADNQPG-		
		ANGADDQPG	27	60
		ANGAGNQPG-		
		ANGADNQPG		61
		ANGAGNQPG-		
		ANGADDQPG		62
		APGANQEGGAA-		
		APGANQEGGAA	28	63
		ANGAGNQPGAN-		
		GAGDQPGANGA-		
		DNQPGANGADD-		
		QPG		64
berghi	CS	DPPPPNPN-		
		DPPPPNPN	2	65
yoelli	CS	(QGPGAP) ₄		66
Streptococcus sobrinus	AgI/II	KPRPIYEA-		
		KLAQNQK	16	67
		AKADYEAK-		
		LAQYEKDL		68
Shigella flexneri	Invasin	KDRTLIEQK	18	69
Respiratory syncytia virus (RSV)	G	CSICSMNPT-		
		CWAICK	19	70
Entamoeba histolytica	lectin	VECASTVCQNDN-		
		SCPIIADVEKCNQ	21	71
Schistosoma japonicum	para	DLQSEISLSLE-		
		NGELIRRAKSA-		
		ESLASELQRRVD	22	72
Schistosoma mansoni	para	DLQSEISLSLE-		
		NSELIRRAKAA-		
		ESLASDLQRRVD	22	73
Bovine Inhibin α_c subunit		STPPLPWPW-		
		SPAALRLLQ-		
		RPPEEPAA	30	74

membrane-anchored glycoprotein

Escherichia coli

Alzheimer's disease

Neisseria meningitidis

- 41 -

MFTPPT	287
MINHRGYWV	288
MGEFCINHRGYWVCGDPA	289

*Citations to published epitopes are provided following Table B.

In the above influenza A M2 sequence of SEQ ID NO: 32,

residues X₁ through X₈ are absent or present, and when present are the residues naturally present in the M2 protein sequence that are methionine, serine, leucine, leucine, threonine, glutamic acid, valine, and glutamic acid, respectively, with the proviso that when one subscripted X residue is present, any remaining subscripted X with a higher subscript number up to 8 is also present,

residues X₁₅ and X₁₆ are present or absent, and when present are tryptophan and glycine, respectively,

residues X₁₇ and X₁₉ are present or absent, and when present are independently cysteine, serine, or alanine,

residue X₁₈ is present or absent, and when present is arginine, and

residues X₂₀ through X₂₄ are present or absent, and when present are the residues naturally present in the M2 protein sequence that are asparagine, aspartic acid, serine, serine and aspartic acid respectively, with the proviso that when one subscripted X residue is present, any remaining subscripted X residue with a lower subscript number down to 15 is also present.

[illegible]

THE

As already noted, a heterologous linker for a conjugated epitope is peptide-bonded at a position in the HBc sequence between amino acid residues 76 and 85. As was the case for the heterologous epitope, the HBc sequence of residues 76 to 85 is

preferably present, but interrupted by the heterologous linker for a conjugated epitope. This chimera preferably includes the HBc sequence of position 4 through at least position 140, plus a cysteine residue near the N-terminus of the chimera protein. More preferably, the HBc sequence of positions 1 through 149 are present, but interrupted between residues 76 and 85 by the heterologous linker for a conjugated epitope, and the chimera molecule contains a C-terminal cysteine.

The heterologous linker for a conjugated epitope is most preferably a lysine (K) residue. Glutamic or aspartic acid, tyrosine and cysteine residues can also be used as linker residues, as can tyrosine and cysteine residues. It is noted that more than one linker can be present such as a sequence of three lysines, but such use is not preferred because heterogeneous conjugates can be formed from such use in which the conjugated hapten is bonded to one linker in a first chimera and to a different linker in a second chimera molecule. U.S. Patent No. 6,231,864 B1 discloses HBc chimera molecules containing one or more linking residues, but lacking a stabilizing N-terminal cysteine residue.

It is also noted that a heterologous epitope sequence present in a contemplated HBc chimera can also be separated from the HBc sequence residues by a "flexible linker arm" on one or both sides of (flanking) the heterologous immunogenic (epitope) sequence. This is particularly the case where the heterologous immunogenic sequence is greater than about 30 amino acid residues long. Exemplary flexible linker arm sequences typically contain about

4 to about 10 glycine residues that are thought to permit the inserted sequence to "bulge" outwardly from the otherwise bulging loop sequence and add further stability to the construct. Illustrative flexible linker arm sequences are disclosed in Kratz et al. (March 1999) *Proc. Natl. Acad. Sci., U.S.A.*, 96:1915-1920 and are exemplified by the amino acid residue sequences:

GGGSGGGGT SEQ ID NO:126

GGGSGGGG SEQ ID NO:127

As was noted previously, Domain III constitutes the sequence of HBc from position 86 through position 135. Consequently, the sequence of the illustrative chimeras discussed above for Domains I and II, can be extended so that the first-discussed chimera has the sequence of HBc from position 84 through position 140, and the second-discussed chimera has the sequence of HBc from position 79 through position 140.

Domain IV is a sequence that (i) includes a HBc sequence from position 136 through 140 and optionally through position 149, (ii) contains zero up to three cysteine residues, and (iii) up to about 100 amino acid residues in a sequence heterologous to HBc at position 150 to the C-terminus, with the proviso that Domain IV contain at least 5 amino acid residues of the HBc sequence from position 136 through 140. The Domain IV sequence heterologous to HBc more preferably contains up to about 50 amino acid residues, and most preferably contains up to about 25 residues. The Domain IV sequence can thus

be substantially any sequence, except the C-terminal HBc sequence from position 150 to the C-terminus.

The length of the Domain IV sequence can be five residues; i.e., the residue of position 136 through 140, up to about 100 amino acid residues including up to a total of three cysteines, with the length being sufficient so that a contemplated chimeric protein has a total length of about 135 to about 515 residues, and more preferably up to about 460 residues, and most preferably up to about 435 amino acid residues. Where an epitope is peptide-bonded to Domains I or II contains up to about 30 or about 50 residues, respectively, as is preferred for those epitopes, more preferred lengths of the chimer molecule, including the Domain IV epitope, are about 175 to about 240 residues. Particularly preferred chimer molecules containing two heterologous epitopes have a length of about 190 to about 210 residues. Freedom of the resulting particle from nucleic acid-binding is determined by determination of the 280/260 absorbance ratio as discussed previously.

The Domain IV sequence can include zero up to three Cys residues. When present, it is preferred that the one or more Cys residues be at or within about five amino acid residues of the C-terminus of the chimeric protein molecule. In addition, when more than one Cys residue is present in a Domain IV sequence, it is preferred that those Cys residues be adjacent to each other.

It is preferred that the Domain IV sequence constitute a T cell epitope, a plurality of T cell epitopes that are the same or different or an additional B cell epitope for the organism against which a contemplated chimer is intended to be used as

an immunogen. Exemplary Domain IV T cell epitope sequences are provided in Table B, below, as in Table A, with illustrative added C-terminal cysteine residues underlined,.

Table B
T Cell Epitopes

<u>Organism</u>	<u>Gene</u>	<u>Sequence*</u>	<u>Citation</u>	SEQ <u>ID NO</u>
HIV	P24	GPKEPFRDY- VDRFYKC	3	128
<i>Corynebacterium diphtheriae</i>	toxin	FQVVHNSYN- RPAYSPGC	5	129
<i>Borrelia burgdorferi</i>	ospA	VEIKEGTVTLKRE- IDKNGKVTVSLC	6	130
		TLSKNISKSG- EVSVELNDC	7	131
Influenza Virus A8/PR8	HA	SSVSSFERFEC	8	132
		LIDALLGDPC	32	133
		TLIDALLGC	32	134
<i>Trypanosoma cruzi</i>		SHNFTLVASVII- EEAPSGNTC	13	135
<i>Plasmodium falciparum</i>	MSP1	SVQIPKVPYPNGIVYC	15	136
		DFNHYYTLKTGLEADC		137
		PSDKHIEQYKKI-	23	
		KNSISC		138
		EYLNKIQNSLST-	26	
		EWSPCSVT		139
<i>P. vivax</i>		YLDKVRATVGTE-		
		WTPCSVT		140
<i>P. yoelii</i>		EFVKQISSQLTE-		
		EWSQCSVT		141

<i>Streptococcus</i>				
<i>sobrinus</i>	AgI/II	KPRPIYEAKL-		
		AQNQKC	16	142
		AKADYEAKLA-		
		QYEKDL <u>C</u>		143
LCMV (lymphocytic choriomeningitis virus)				
	NP	RPQASGVYM-		
		GNLTAQ <u>C</u>	17	144
<i>Clostridium</i>				
<i>tetani</i>	tox			
		QYIKANSKFIG-		
		ITEL <u>C</u>	20	145
<i>Neisseria meningitidis</i>				
	PorB	AIWQVEQKASIAGTDSGWC		146
		NYKNGGFFVQYGGAYKRHC		147
		HNSQTEVAATLAYRFGNVC		148
	PorB	TPRVSYAHGFKGLVDDADC		149
		RFGNAVPRISYAHGFD <u>F</u> IC		150
		AFKYARHANVGRNAFELFC		151
		SGAWLKRNTGIGNYTQINAC		152
		AGEFGTLRAGRANQC		153
		IGNYTQINAASVGLRC		154
		GRNYQLQLTEQPSRTC		155
		SGSVQFVPAQNSKSAC		156
		HANVGRDAFNLFLLGC		157
		LGRIGDDDEAKGTDPC		158
		SVQFVPAQNSKSAYKC		159
		NYAFKYAKHANVGRDC		160
		AHGFD <u>F</u> IERGKKGENC		161
		GVDYDFSKRTSAIVSC		162
		HDDMPVSVRYDSPDFC		163
		RFGNAVPRISYAHGFD <u>F</u> IERGKKGENC		164
		NYAFKYAKHANVGRDAFNLFLLGC		165
		SGAWLKRNTGIGNYTQINAASVGLRC		166
		SGSVQFVPAQNSKSAYTPAC		167
	OpaB	TGANNTSTVSDYFRNRITC		168
		IYDFKLNDKFDKFKPYIGC		169
	Opa-5d	LSAIYDFKLNDKFKPYIGC		170
	Opac	NGWYINPWSEVKFDLNSRC		171

*Underlined C (C) is not from the native sequence.

Citations:

1. EPO 786 521A.
2. WO 98/07320.
3. US No. 5,639,854.
4. US No. 4,544,500.
5. EPO 399001 B1.
6. Bockenstedt et al. (1996) *J. Immunol.*, 157, 12:5496.
7. Zhong et al. (1996) *Eur. J. Immunol.*, 26, 11:2749.

8. Brumeanu et al. (1996) *Immunotechnology*, 2, 2:85.
9. Hill et al. (1997) *Infect. Immun.*, 65, 11:4476.
10. EPO 432 220 B1.
11. WO 98/06851.
12. Kelly et al. (1997) *Clin. Exp. Immunol.*, 110, 2:285.
13. Kahn et al. (1997) *J. Immunol.*, 159, 9:4444.
14. WO 97/18475.
15. Ohta et al. (1997) *Int. Arch. Allergy Immunol.*, 114,1:15.
16. Staffileno et al. (1990) *Arch. Oral Biol.*, 35: Suppl. 47S.
17. Saron et al. (1997) *Proc. Natl. Acad. Sci. USA*, 94,7:3314.
18. Corthesy et al. (1996) *J. Biol. Chem.*, 271, 52:33670.
19. Bastien et al. (1997) *Virology*, 234, 1:118.
20. Yang et al. (1997) *Vaccine*, 15, 4:377.
21. Lotter et al. (1997) *J. Exp. Med.*, 185, 10:1793.
22. Nara et al. (1997) *Vaccine* 15, 1:79.
23. U.S. No. 4,886,782.
24. Zavala et al. (1985) *Science*, 228:1436.
25. Schodel et al. (1994) *J. Exper. Med.*, 180:1037.
26. Calvo-Calle et al. (1997) *J. Immunol.* 159, 3:1362.
27. Qari et al. (1992) *Mol. Biochem. Parasitol.*, 55(1-2):105.
28. Qari et al. (1993) *Lancet*, 341(8848):780.
29. Neirynck et al. (Oct 1999) *Nature Med.*, 5(10):1157-1163.
30. Thompson et al. (1994) *Eur.J. Biochem.*, 226(3):751-764.
31. Wilson et al. (2000) *Science*, 287:1664-1666.
32. Brown et al. (1993) *J. Virol.*, 67(5):2887-2893.
33. U.S. No. 4,886,663.
34. Schenk et al. (Jul 8, 1999) *Nature*, 400(6740):116-117.
35. Slepishkin et al. (1995) *Vaccine*, 13(15):1399-1402.

The amino acid sequence of HBC from residue position 4 through at least position 140 is preferably present in a contemplated chimer molecule and particle. The sequence from position 2 through position 149 is more preferably present. A B cell epitope is preferably present between residues 76 and 85 and at least a single cysteine residue at or near the N-terminus in Domain I as already noted, a T cell epitope that can include a cysteine residue can also be present as a C-terminal addition to the HBC

sequence. A contemplated recombinant HBc chimer is substantially free of bound nucleic acid. A contemplated chimer particle that contains an added Cys residue at or near the N-terminus of the molecule is also more stable after formation than is a similar particle that does not contain that added Cys.

A contemplated recombinant HBc chimer molecule is typically present and is used as a self-assembled particle. These particles are comprised of 180 to 240 chimer molecules (90 or 120 dimer pairs), usually 240 chimer molecules, that separate into protein molecules in the presence of disulfide reducing agents such as 2-mercaptoethanol, and the individual molecules are therefore thought to be bound together into the particle primarily by disulfide bonds.

Although not wishing to be bound by theory, it is believed that the observed enhanced stability and in some cases enhanced expression for a contemplated HBc chimer is due to the formation of an N-terminal cystine disulfide bond between chimer protein molecules of the particles. Regardless of whether present as a cysteine or a cystine, the N-terminal cysteine(s) residue is referred to as a cysteine inasmuch as that is the residue coded-for by the codon present in the nucleic acid from which the protein and assembled particle is expressed.

These particles are similar to the particles observed in patients infected with HBV, but these particles are non-infectious. Upon expression in various prokaryotic and eukaryotic hosts, the individual recombinant HBc chimer molecules assemble in the host into particles that can be readily

harvested from the host cells, and purified, if desired.

As noted before, the HBc immunodominant loop is usually recited as being located at about positions 75 to 85 from the amino-terminus (N-terminus) of the intact protein. The heterologous B cell epitope-containing sequence of Domain II is placed into that immunodominant loop sequence. That placement substantially eliminates the HBc immunogenicity of the HBc loop sequence, while presenting the heterologous sequence or linker residue in an extremely immunogenic position in the assembled chimer particles.

In addition to the before-discussed N- and C-truncations, insertion of various epitopes and spacers, a contemplated chimer molecule can also contain conservative substitutions in the amino acid residues that constitute HBc Domains I, II, III and IV. Conservative substitutions are as defined before. An illustrative conservative substitution is seen in the replacement of residues at positions 2 and 3 (aspartic acid and isoleucine; DI) by glutamic acid and leucine (EL) residues that are encoded by an EcoRI restriction site used to add nucleic acids that code for a desired N-terminal epitope, including an N-terminal cysteine residue.

More rarely, a "nonconservative" change, e.g., replacement of a glycine with a tryptophan is contemplated. Analogous minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity or particle formation can be found using computer programs well known in

the art, for example LASERGENE software (DNASTAR Inc., Madison, Wis.)

The HBc portion of a chimer molecule of the present invention; i.e., the portion having the HBc sequence that has other than a sequence or residue of an added epitope, linker, flexible linker arm or heterologous residue(s) that are a restriction enzyme artifact, most preferably has the amino acid residue sequence at positions 2 through 149 of subtype ayw that is shown in Fig. 1 (SEQ ID NO:1), less any portion or portions of the subtype ayw sequence that are absent because of truncation at one or both termini. Somewhat less preferred are the corresponding amino acid residue sequences of subtypes adw, adw2 and adyw that are also shown in Fig. 1 (SEQ ID NOS:2, 3 AND 4). Less preferred still are the sequences of woodchuck and ground squirrel at aligned positions 2 through 149 that are the last two sequences of Fig 1 (SEQ ID NOS:5 and 6). As noted elsewhere, portions of different sequences from different mammalian HBc proteins can be used together in a single chimera.

When the HBc portion of a chimera molecule of the present invention as above described has other than a sequence of a mammalian HBc molecule corresponding to positions 2 through 149, no more than about 20 percent of the amino acid residues are substituted as compared to SEQ ID NO:1 from position 2 through 149. It is preferred that no more than about 10 percent, and more preferably no more than about 5 percent, and most preferably no more than about 3 percent of the amino acid residues are substituted as compared to SEQ ID NO:1 from position 2 through 149.

A contemplated chimera of 149 HBc residues can therefore contain up to about 30 residues that are different from those of SEQ ID NO:1 at positions 2 through 149, and preferably about 15 residues. More preferably, about 7 or 8 residues are different from the ayw sequence (SEQ ID NO:1) at residue positions 2-149, and most preferably about 4 or 5 residues are different. Substitutions, other than in the immunodominant loop of Domain II or at the termini, are preferably in the non-helical portions of the chimera molecule and are typically between residues 2 to about 15 and residues 24 to about 50 to help assure particle formation. See, Koschel et al., *J. Virol.*, **73(3)**:2153-2160 (Mar. 1999).

Where a HBc sequence is truncated at the C-terminus beyond position 149 or at the N-terminus, or contains one or more deletions in the immunogenic loop, the number of substituted residues is proportionally different because the total length of the sequence is less than 149 residues. Deletions elsewhere in the molecule are considered conservative substitutions for purposes of calculation.

Chimer Preparation

A contemplated chimeric HBc immunogen is typically prepared using the well-known techniques of recombinant DNA technology. Thus, sequences of nucleic acid that encode particular polypeptide sequences are added to and deleted from the precursor sequence that encodes HBc to form a nucleic acid that encodes a contemplated chimera.

An illustrative contemplated chimeric immunogen typically utilizes a cysteine residue present in the influenza A M2 sequence as the N-

$$= \frac{1}{\sqrt{2}} \begin{pmatrix} -\frac{\sqrt{2}}{2} & \frac{1}{2} \\ \frac{\sqrt{2}}{2} & \frac{1}{2} \end{pmatrix} \begin{pmatrix} \frac{1}{2} & \frac{\sqrt{2}}{2} \\ \frac{\sqrt{2}}{2} & \frac{1}{2} \end{pmatrix} = \frac{1}{2} \begin{pmatrix} -\frac{\sqrt{2}}{2} & \frac{1}{2} \\ \frac{\sqrt{2}}{2} & \frac{1}{2} \end{pmatrix} \begin{pmatrix} \frac{1}{2} & \frac{\sqrt{2}}{2} \\ \frac{\sqrt{2}}{2} & \frac{1}{2} \end{pmatrix}$$

1944-1945

1944-1945

1944-1945

[illegible]

[REDACTED]

- 56 -

Insertion is therefore generally preferred. In an illustrative example of the insertion strategy, site-directed mutagenesis is used to create two restriction sites adjacent to each other and between codons encoding adjacent amino acid residues, such as those at residue positions 78 and 79. This technique adds twelve base pairs that encode four amino acid residues (two for each restriction site) between formerly adjacent residues in the HBc loop.

Upon cleavage with the restriction enzymes, ligation of the DNA coding for the heterologous B cell epitope sequence and expression of the DNA to form HBc chimeres, the HBc loop amino acid sequence is seen to be interrupted on its N-terminal side by the two residues encoded by the 5' restriction site, followed toward the C-terminus by the heterologous B-cell epitope sequence, followed by two more heterologous, non-loop residues encoded by the 3' restriction site and then the rest of the loop sequence. This same strategy can be used for insertion into Domain I of an N-terminal cysteine or N-terminal sequence as was reported in Neirynck et al., (October 1999) *Nature Med.*, 5(10):1157-1163 or for insertion into Domain IV of a T cell epitope or one or more cysteine residues. A similar strategy using an insertion between residues 82 and 83 is reported in Schodel et al., (1990) F. Brown et al. eds., *Vaccines 90*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp.193-198.

More specifically, a DNA sequence that encodes a C-terminal truncated HBc sequence (HBc149) is engineered to contain adjacent EcoRI and SacI sites between residues 78 and 79. Cleavage of that DNA with both enzymes provides one fragment that

encodes HBc positions 1-78 3'-terminated with an EcoRI sticky end, whereas the other fragment has a 5'-terminal SacI sticky end and encodes residues of positions 79-149. Ligation of a synthetic nucleic acid having a 5' AATT overhang followed by a sequence that encodes a desired B cell epitope and a AGCT 3'overhang provides a HBc chimer sequence that encodes that B cell epitope flanked on each side by two heterologous residues [GlyIle (GI) and GluLeu (EL), respectively] between residues 78 and 79, while usually destroying the EcoRI site and preserving the SacI site.

A similar strategy for insertion of a cysteine-containing sequence in Domain IV, such as a malarial T cell epitope that contains the P. *falciparum* CS protein sequence from position 326 through position 345 and is referred to herein as PF/CS326-345 (Pf-UTC). Here, EcoRI and HindIII restriction sites are engineered into the HBc DNA sequence after amino acid residue position 149. After digestion with EcoRI and HindIII, a synthetic DNA having the above AATT 5'overhang followed by a T cell epitope-encoding sequence, one or more stop codons and a 3' AGCT overhang were ligated into the digested sequence to form a sequence that encoded HBc residues 1-149 followed by two heterologous residues (GI), the stop codon and the HindIII site.

PCR amplification using a forward primer having a SacI restriction site followed by a sequence encoding HBc beginning at residue position 79, followed by digestion with SacI and HindIII provided a sequence encoding HBc positions 79-149 plus the two added residues and the T cell epitope at the C-terminus. Digestion of the construct with SacI and

ligation provides the complete gene encoding a desired recombinant HBc chimer immunogen having the sequence, from the N-terminus, of HBc positions 1-78, two added residues, the malarial B cell epitope, two added residues, HBc positions 79-149, two added residues, and the T cell epitope that is shown in Fig. 2C.

Similar techniques can be used to place a heterologous linker residue for conjugation of a B cell epitope into the loop region sequence. Contemplated linker residues include lysine (Lys), which is particularly preferred, aspartic acid (Asp), glutamic acid (Glu), cysteine (Cys) and tyrosine (Tyr).

It is noted that the amino acid residue sequence shown in SEQ ID NO:1 contains a Glu and an Asp residue at positions 77 and 78. Nonetheless, introduction of an additional, heterologous, carboxyl-containing residue is still contemplated. The chemical reactivity of the existing glutamic and aspartic acids may be reduced by other factors. For example, it is known in the art that a neighboring proline, such as that found at position 79, can neutralize and thereby reduce the chemical reactivity of a proximal carboxyl group.

Here, using the first noted insertion strategy, five heterologous residues are placed into the loop sequence; one that is the heterologous linker residue for conjugating a B cell epitope and two residues adjacent on either side of that one residue that are themselves also adjacent to loop sequence residues and are an expression product of the inserted restriction sites (restriction enzyme artifacts). It is noted that one can also use site-

directed mutagenesis to add a single codon into the HBc loop sequence that encodes the heterologous linker residue for a B cell epitope.

It is noted that the preferred use of two heterologous residues on either side of (flanking) a B cell or T cell epitope is a matter of convenience. As a consequence, one can also use zero to three or more added residues that are not part of the HBc sequence on either or both sides of an inserted sequence. One or both ends of the insert and HBc nucleic acid can be "chewed back" with an appropriate nuclease (e.g. S1 nuclease) to provide blunt ends that can be ligated together. Added heterologous residues that are neither part of the inserted B cell or T cell epitopes nor a part of the HBc sequence are not counted in the number of residues present in a recited Domain, unless those residues are conservative replacements for residues already present, as where the residues GluLeu replace AspIle in some of the constructs discussed hereinafter.

It is also noted that one can also synthesize all or a part of a desired recombinant HBc chimer nucleic acid using well-known synthetic methods as is discussed and illustrated in U. S. Patent No. 5,656,472 for the synthesis of the 177 base pair DNA that encodes the 59 residue ribulose bis-phosphate carboxylase-oxygenase signal peptide of *Nicotiana tabacum*. For example, one can synthesize Domains I and II with a blunt or a "sticky end" that can be ligated to Domains III and IV to provide a construct that expresses a contemplated HBc chimer that contains zero added residues to the N-terminal side of the B cell epitope and zero to three added

residues on the C-terminal side or at the Domain II/III junction or at some other desired location.

An alternative insertion technique was reported in Clarke et al. (1991) F. Brown et al. eds., *Vaccines 91*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp.313-318. Here, taking advantage of the degeneracy of the genetic code, those workers engineered a single restriction site corresponding to residues 80 and 81 that encoded the original residues present at those positions. Their expressed HBc chimeres thereby contained no restriction site-encoded residues, and contained the residues of the HBc loop immediately adjacent to the inserted sequence.

A nucleic acid sequence (segment) that encodes a previously described HBc chimera molecule or a complement of that coding sequence is also contemplated herein. Such a nucleic acid segment is present in isolated and purified form in some preferred embodiments.

In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the gene that codes for the protein. Thus, through the well-known degeneracy of the genetic code additional DNAs and corresponding RNA sequences (nucleic acids) can be prepared as desired that encode the same chimera amino acid residue sequences, but are sufficiently different from a before-discussed gene sequence that the two sequences do not hybridize at high stringency, but do hybridize at moderate stringency.

High stringency conditions can be defined as comprising hybridization at a temperature of about

50°-55°C in 6XSSC and a final wash at a temperature of 68°C in 1-3XSSC. Moderate stringency conditions comprise hybridization at a temperature of about 50°C to about 65°C in 0.2 to 0.3 M NaCl, followed by washing at about 50°C to about 55°C in 0.2X SSC, 0.1% SDS (sodium dodecyl sulfate).

A nucleic sequence (DNA sequence or an RNA sequence) that (1) itself encodes, or its complement encodes, a chimer molecule whose HBc portion from residue position 4 through 136, when present, is that of SEQ ID NOs: 1, 2, 3, 4, 5 or 6 and (2) hybridizes with a DNA sequence of SEQ ID NOs: 172, 173, 174, 175, 176 or 177 at least at moderate stringency (discussed above); and (3) whose HBc sequence shares at least 80 percent, and more preferably at least 90 percent, and even more preferably at least 95 percent, and most preferably 100 percent identity with a DNA sequence of SEQ ID NOs: 172, 173, 174, 175, 176 and 177, is defined as a DNA variant sequence. As is well-known, a nucleic acid sequence such as a contemplated nucleic acid sequence is expressed when operatively linked to an appropriate promoter in an appropriate expression system as discussed elsewhere herein.

An analog or analogous nucleic acid (DNA or RNA) sequence that encodes a contemplated chimer molecule is also contemplated as part of this invention. A chimer analog nucleic acid sequence or its complementary nucleic acid sequence encodes a HBc amino acid residue sequence that is at least 80 percent, and more preferably at least 90 percent, and most preferably is at least 95 percent identical to the HBc sequence portion from residue position 4 through residue position 136 shown in SEQ ID NOs: 1,

2, 3, 4, 5 or 6. This DNA or RNA is referred to herein as an "analog of" or "analogous to" a sequence of a nucleic acid of SEQ ID NOs: 172, 173, 174, 175, 176 and 177, and hybridizes with the nucleic acid sequence of SEQ ID NOs: 172, 173, 174, 175, 176 and 177 or their complements herein under moderate stringency hybridization conditions. A nucleic acid that encodes an analogous sequence, upon suitable transfection and expression, also produces a contemplated chimera.

Different hosts often have preferences for a particular codon to be used for encoding a particular amino acid residue. Such codon preferences are well known and a DNA sequence encoding a desired chimera sequence can be altered, using *in vitro* mutagenesis for example, so that host-preferred codons are utilized for a particular host in which the enzyme is to be expressed. In addition, one can also use the degeneracy of the genetic code to encode the HBc portion of a sequence of SEQ ID NOs: 172, 173, 174, 175, 176 or 177 that avoids substantial identity with a DNA of SEQ ID Nos: 1, 2, 3, 4, 5 or 6 or their complements. Thus, a useful analogous DNA sequence need not hybridize with the nucleotide sequences of SEQ ID NOs: 172, 173, 174, 175, 176 or 177 or a complement under conditions of moderate stringency, but can still provide a contemplated chimera molecule.

A recombinant nucleic acid molecule such as a DNA molecule, comprising a vector operatively linked to an exogenous nucleic acid segment (e.g., a DNA segment or sequence) that defines a gene that encodes a contemplated chimera, as discussed above, and a promoter suitable for driving the expression of

the gene in a compatible host organism, is also contemplated in this invention. More particularly, also contemplated is a recombinant DNA molecule that comprises a vector comprising a promoter for driving the expression of the chimera in host organism cells operatively linked to a DNA segment that defines a gene for the HBc portion of a chimera or a DNA variant that has at least 90 percent identity to the chimera gene of SEQ ID NOs: 172, 173, 174, 175, 176 or 177 and hybridizes with that gene under moderate stringency conditions.

Further contemplated is a recombinant DNA molecule that comprises a vector containing a promoter for driving the expression of a chimera in host organism cells operatively linked to a DNA segment that is an analog nucleic acid sequence that encodes an amino acid residue sequence of a HBc chimera portion that is at least 80 percent identical, more preferably 90 percent identical, and most preferably 95 percent identical to the HBc portion of a sequence of SEQ ID NOs: 1, 2, 3, 4, 5 or 6. That recombinant DNA molecule, upon suitable transfection and expression in a host cell, provides a contemplated chimera molecule.

It is noted that because of the 30 amino acid residue N-terminal sequence of ground squirrel HBc does not align with any of the other HBc sequences, that sequence and its encoding nucleic acid sequences and their complements are not included in the above percentages of identity, nor are the portions of nucleic acid that encode that 30-residue sequence or its complement used in hybridization determinations. Similarly, sequences that are truncated at either or both of the HBc N- and C-

termini are not included in identity calculations, nor are those sequences in which residues of the immunodominant loop are removed for insertion of a heterologous epitope. Thus, only those HBc-encoding bases or HBc sequence residues that are present in a chimer molecule are included and compared to an aligned nucleic acid or amino acid residue sequence in the identity percentage calculations.

Inasmuch as the coding sequences for the gene disclosed herein is illustrated in SEQ ID NOs: 172, 173, 174, 175, 176 and 177, isolated nucleic acid segments, preferably DNA sequences, variants and analogs thereof can be prepared by *in vitro* mutagenesis, as is well known in the art and discussed in Current Protocols In Molecular Biology, Ausabel et al. eds., John Wiley & Sons (New York: 1987) p. 8.1.1-8.1.6, that begin at the initial ATG codon for a gene and end at or just downstream of the stop codon for each gene. Thus, a desired restriction site can be engineered at or upstream of the initiation codon, and at or downstream of the stop codon so that other genes can be prepared, excised and isolated.

As is well known in the art, so long as the required nucleic acid, illustratively DNA sequence, is present, (including start and stop signals), additional base pairs can usually be present at either end of the segment and that segment can still be utilized to express the protein. This, of course, presumes the absence in the segment of an operatively linked DNA sequence that represses expression, expresses a further product that consumes the enzyme desired to be expressed, expresses a product that consumes a wanted reaction product produced by that

desired enzyme, or otherwise interferes with expression of the gene of the DNA segment.

Thus, so long as the DNA segment is free of such interfering DNA sequences, a DNA segment of the invention can be about 500 to about 15,000 base pairs in length. The maximum size of a recombinant DNA molecule, particularly an expression vector, is governed mostly by convenience and the vector size that can be accommodated by a host cell, once all of the minimal DNA sequences required for replication and expression, when desired, are present. Minimal vector sizes are well known. Such long DNA segments are not preferred, but can be used.

DNA segments that encode the before-described chimera can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al. (1981) *J. Am. Chem. Soc.*, **103**:3185. Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. However, DNA segments including sequences discussed previously are preferred.

A contemplated HBC chimera can be produced (expressed) in a number of transformed host systems, typically host cells although expression in acellular, *in vitro*, systems is also contemplated. These host cellular systems include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g. baculovirus); plant cell systems transformed with

virus expression vectors (e.g. cauliflower mosaic virus; tobacco mosaic virus) or with bacterial expression vectors (e.g., Ti plasmid); or appropriately transformed animal cell systems such as CHO, VERO or COS cells. The invention is not limited by the host cell employed.

DNA segments containing a gene encoding the HBc chimera are preferably obtained from recombinant DNA molecules (plasmid vectors) containing that gene. Vectors capable of directing the expression of a chimera gene into the protein of a HBc chimera is referred to herein as an "expression vector".

An expression vector contains expression control elements including the promoter. The chimera-coding gene is operatively linked to the expression vector to permit the promoter sequence to direct RNA polymerase binding and expression of the chimera-encoding gene. Useful in expressing the polypeptide coding gene are promoters that are inducible, viral, synthetic, constitutive as described by Poszkowski et al. (1989) *EMBO J.*, 3:2719 and Odell et al. (1985) *Nature*, 313:810, as well as temporally regulated, spatially regulated, and spatiotemporally regulated as given in Chua et al. (1989) *Science*, 244:174-181.

One preferred promoter for use in prokaryotic cells such as *E. coli* is the Rec 7 promoter that is inducible by exogenously supplied nalidixic acid. A more preferred promoter is present in plasmid vector JHEX25 (available from Promega Corp., Madison WI) that is inducible by exogenously supplied isopropyl- β -D-thiogalactopyranoside (IPTG). A still more preferred promoter, the tac promoter, is present in plasmid vector pKK223-3 and is also inducible by exogenously supplied IPTG. The pKK223-3

plasmid can be successfully expressed in a number of *E. coli* strains, such as XL-1, TB1, BL21 and BLR, using about 25 to about 100 μ M IPTG for induction. Surprisingly, concentrations of about 25 to about 50 μ M IPTG have been found to provide optimal results in 2 L shaker flasks and fermentors.

Several strains of *Salmonella* such as *S. typhi* and *S. typhimurium* and *S. typhimurium-E. coli* hybrids have been used to express immunogenic transgenes including prior HBc chimer particles both as sources of the particles for use as immunogens and as live, attenuated whole cell vaccines and inocula, and those expression and vaccination systems can be used herein. See, U.S. Patent No. 6,024,961; U.S. Patent No. 5,888,799; U.S. Patent No. 5,387,744; U.S. Patent No. 5,297,441; Ulrich et al., (1998) *Adv. Virus Res.*, **50**:141-182; Tacket et al., (Aug 1997) *Infect. Immun.*, **65**(8):3381-3385; Schodel et al., (Feb 1997) *Behring Inst. Mitt.*, **98**:114-119; Nardelli-Haeffliger et al., (Dec 1996) *Infect. Immun.*, **64**(12):5219-5224; Londono et al., (Apr 1996) *Vaccine*, **14**(6):545-552, and the citations therein.

Expression vectors compatible with eukaryotic cells, such as those compatible with yeast cells or those compatible with cells of higher plants or mammals, are also contemplated herein. Such expression vectors can also be used to form the recombinant DNA molecules of the present invention. Vectors for use in yeasts such as *S. cerevisiae* or *Pichia pastoris* can be episomal or integrating, as is well known. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Normally, such vectors contain one or more convenient restriction sites for

insertion of the desired DNA segment and promoter sequences. Optionally, such vectors contain a selectable marker specific for use in eukaryotic cells. Exemplary promoters for use in *S. cerevisiae* include the *S. cerevisiae* phosphoglyceric acid kinase (PGK) promoter and the divergent promoters GAL 10 and GAL 1, whereas the alcohol oxidase gene (AOX1) is a useful promoter for *Pichia pastoris*.

For example, to produce chimeras in the methylotrophic yeast, *P. pastoris*, a gene that encodes a desired chimera is placed under the control of regulatory sequences that direct expression of structural genes in *Pichia*. The resultant expression-competent forms of those genes are introduced into *Pichia* cells.

More specifically, the transformation and expression system described by Cregg et al. (1987) *Biotechnology*, 5:479-485; (1987) *Molecular and Cellular Biology*, 12:3376-3385 can be used. A gene for a chimera V12.Pf3.1 is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. The gene and its flanking regulatory regions are then introduced into a plasmid that carries both the *P. pastoris* HIS4 gene and a *P. pastoris* ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within *P. pastoris* cells [Cregg et al. (1987) *Molecular and Cellular Biology*, 12:3376-3385].

The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in *E. coli* cells. The resultant plasmid carrying a chimera gene, as well as the various additional elements described above, is

illustratively transformed into a his4 mutant of *P. pastoris*; i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described Cregg et al. (1987) *Molecular and Cellular Biology*, 12:3376-3385, to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the chimer protein and the production of chimer particles in *P. pastoris*.

A contemplated chimer gene can also be introduced by integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al. (1987) *Molecular and Cellular Biology*, 12:3376-3385.

Production of chimer particles by recombinant DNA expression in mammalian cells is illustratively carried out using a recombinant DNA vector capable of expressing the chimer gene in Chinese hamster ovary (CHO) cells. This is accomplished using procedures that are well known in the art and are described in more detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratories (1989).

In one illustrative example, the simian virus (SV40) based expression vector, pKSV-10 (Pharmacia Fine Chemicals, Piscataway, NJ), is subjected to restriction endonuclease digestion by NcoI and HindIII. A NcoI/HindIII sequence fragment that encodes the desired HBc chimer prepared as described in Example 1 is ligated into the expression plasmid, which results in the formation of a circular recombinant expression plasmid denominated pSV-Pf.

$$= \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} e^{-t^2} dt = \frac{1}{\sqrt{\pi}} \cdot \sqrt{\pi} = 1$$

1000 - 1000

containing cellular protein is separated on a column as discussed elsewhere herein.

The choice of which expression vector and ultimately to which promoter a chimer-encoding gene is operatively linked depends directly on the functional properties desired, e.g. the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention can direct the replication, and preferably also the expression (for an expression vector) of the chimer gene included in the DNA segment to which it is operatively linked.

In one preferred embodiment, the host that expresses the chimer is the prokaryote, *E. coli*, and a preferred vector includes a prokaryotic replicon; i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell transformed therewith. Such replicons are well known in the art.

Those vectors that include a prokaryotic replicon can also include a prokaryotic promoter region capable of directing the expression of a contemplated HBC chimer gene in a host cell, such as *E. coli*, transformed therewith. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing one or more convenient restriction sites for insertion of a contemplated DNA segment. Typical of such vector plasmids are pUC8, pUC9, and pBR329 available from Biorad Laboratories, (Richmond, CA) and pPL and pKK223-3 available from Pharmacia, Piscataway, NJ.

Typical vectors useful for expression of genes in cells from higher plants and mammals are well known in the art and include plant vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers et al. (1987) *Meth. in Enzymol.*, **153**:253-277 and mammalian expression vectors pKSV-10, above, and pCI-neo (Promega Corp., #E1841, Madison, WI). However, several other expression vector systems are known to function in plants including pCaMVCN transfer control vector described by Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA*, **82**:58-24. Plasmid pCaMVCN (available from Pharmacia, Piscataway, NJ) includes the cauliflower mosaic virus CaMV 35S promoter.

The above plant expression systems typically provide systemic or constitutive expression of an inserted transgene. Systemic expression can be useful where most or all of a plant is used as the source to a contemplated chimer molecule or resultant particles or where a large part of the plant is used to provide an oral vaccine. However, it can be more efficacious to express a chimer molecule or particles in a plant storage organ such as a root, seed or fruit from which the particles can be more readily isolated or ingested.

One manner of achieving storage organ expression is to use a promoter that expresses its controlled gene in one or more preselected or predetermined non-photosynthetic plant organs. Expression in one or more preselected storage organs with little or no expression in other organs such as roots, seed or fruit versus leaves or stems is referred to herein as enhanced or preferential expression. An exemplary promoter that directs

expression in one or more preselected organs as compared to another organ at a ratio of at least 5:1 is defined herein as an organ-enhanced promoter. Expression in substantially only one storage organ and substantially no expression in other storage organs is referred to as organ-specific expression; i.e., a ratio of expression products in a storage organ relative to another of about 100:1 or greater indicates organ specificity. Storage organ-specific promoters are thus members of the class of storage organ-enhanced promoters.

Exemplary plant storage organs include the roots of carrots, taro or manioc, potato tubers, and the meat of fruit such as red guava, passion fruit, mango, papaya, tomato, avocado, cherry, tangerine, mandarin, palm, melons such cantaloupe and watermelons and other fleshy fruits such as squash, cucumbers, mangos, apricots, peaches, as well as the seeds of maize (corn), soybeans, rice, oil seed rape and the like.

The CaMV 35S promoter is normally deemed to be a constitutive promoter. However, recent research has shown that a 21-bp region of the CaMV 35S promoter, when operatively linked into another, heterologous usual green tissue promoter, the rbcS-3A promoter, can cause the resulting chimeric promoter to become a root-enhanced promoter. That 21-bp sequence is disclosed in U.S. Patent No. 5,023,179. The chimeric rbcS-3A promoter containing the 21-bp insert of U.S. Patent No. 5,023,179 is a useful root-enhanced promoter herein.

A similar root-enhanced promoter, that includes the above 21 bp segment is the -90 to +8 region of the CAMV 35S promoter itself. U.S. Patent

No. 5,110,732 discloses that that truncated CaMV 35S promoter provides enhanced expression in roots and the radical of seed, a tissue destined to become a root. That promoter is also useful herein.

Another useful root-enhanced promoter is the -1616 to -1 promoter of the oil seed rape (*Brassica napus* L.) gene disclosed in PCT/GB92/00416 (WO 91/13922 published Sep. 19, 1991). *E. coli* DH5.alpha. harboring plasmid pRlambdaS4 and bacteriophage lambda.beta.1 that contain this promoter were deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on Mar. 8, 1990 and have accession numbers NCIMB40265 and NCIMB40266. A useful portion of this promoter can be obtained as a 1.0 kb fragment by cleavage of the plasmid with HaeIII.

A preferred root-enhanced promoter is the mannopine synthase (mas) promoter present in plasmid pKan2 described by DiRita et al. (1987) *Mol. Gen. Genet.*, 207:233-241. This promoter is removable from its plasmid pKan2 as a XbaI-XbaII fragment.

The preferred mannopine synthase root-enhanced promoter is comprised of the core mannopine synthase (mas) promoter region up to position -138 and the mannopine synthase activator from -318 to -213, and is collectively referred to as AmasPmas. This promoter has been found to increase production in tobacco roots about 10- to about 100-fold compared to leaf expression levels.

Another root specific promoter is the about 500 bp 5' flanking sequence accompanying the hydroxyproline-rich glycopeptide gene, HRGPnt3, expressed during lateral root initiation and reported by Keller et al. (1989) *Genes Dev.*, 3:1639-1646.

— Второй этап — обработка и запись информации

1. The first part of the document is a letter from the author to the editor, dated 10/10/1961. The letter discusses the author's interest in the topic of the journal and the importance of the research. The author mentions that the research was conducted in a laboratory setting and that the results are preliminary. The author also mentions that the research was funded by the National Science Foundation.

(

•

abundant in stems and roots. Yang et al. (1990) *Proc. Natl. Acad. Sci., U.S.A.*, **87**:4144-4148. This promoter is thus useful for plant organs such as fleshy fruits like melons, e.g. cantaloupe, or seeds that contain endosperm and for roots that have high levels of phloem cells.

Another exemplary tissue-specific promoter is the lectin promoter, which is specific for seed tissue. The lectin protein in soybean seeds is encoded by a single gene (Le1) that is only expressed during seed maturation and accounts for about 2 to about 5 percent of total seed mRNA. The lectin gene and seed-specific promoter have been fully characterized and used to direct seed specific expression in transgenic tobacco plants. See, e.g., Vodkin et al. (1983) *Cell*, **34**:1023 and Lindstrom et al. (1990) *Developmental Genetics*, **11**:160.

A particularly preferred tuber-specific expression promoter is the 5' flanking region of the potato patatin gene. Use of this promoter is described in Twell et al. (1987) *Plant Mol. Biol.*, **9**:365-375. This promoter is present in an about 406 bp fragment of bacteriophage LPOTI. The LPOTI promoter has regions of over 90 percent homology with four other patatin promoters and about 95 percent homology over all 400 bases with patatin promoter PGT5. Each of these promoters is useful herein. See, also, Wenzler et al. (1989) *Plant Mol. Biol.*, **12**:41-50.

Still further organ-enhanced and organ-specific promoter are disclosed in Benfey et al. (1988) *Science*, **244**:174-181.

Each of the promoter sequences utilized is substantially unaffected by the amount of chimer

molecule or particles in the cell. As used herein, the term "substantially unaffected" means that the promoter is not responsive to direct feedback control (inhibition) by the chimer molecules or particles accumulated in transformed cells or transgenic plant.

Transfection of plant cells using *Agrobacterium tumefaciens* is typically best carried out on dicotyledonous plants. Monocots are usually most readily transformed by so-called direct gene transfer of protoplasts. Direct gene transfer is usually carried out by electroportation, by polyethyleneglycol-mediated transfer or bombardment of cells by microprojectiles carrying the needed DNA. These methods of transfection are well-known in the art and need not be further discussed herein. Methods of regenerating whole plants from transfected cells and protoplasts are also well-known, as are techniques for obtaining a desired protein from plant tissues. See, also, U.S. Patents No. 5,618,988 and 5,679,880 and the citations therein.

A transgenic plant formed using *Agrobacterium* transformation, electroportation or other methods typically contains a single gene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, it is believed that a more accurate name for such a plant is an independent segregant, because the added, exogenous chimer molecule-encoding gene segregates independently during mitosis and meiosis. A

transgenic plant containing an organ-enhanced promoter driving a single structural gene that encodes a contemplated HBc chimeric molecule; i.e., an independent segregant, is a preferred transgenic plant.

More preferred is a transgenic plant that is homozygous for the added structural gene; i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced chimer particle accumulation relative to a control (native, non-transgenic) or an independent segregant transgenic plant. A homozygous transgenic plant exhibits enhanced chimer particle accumulation as compared to both a native, non-transgenic plant and an independent segregant transgenic plant.

It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous (heterologous) genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a chimeric HBc molecule. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

A transgenic plant of this invention thus has a heterologous structural gene that encodes a contemplated chimeric HBc molecule. A preferred transgenic plant is an independent segregant for the added heterologous chimeric HBc structural gene and

can transmit that gene to its progeny. A more preferred transgenic plant is homozygous for the heterologous gene, and transmits that gene to all of its offspring on sexual mating.

Inasmuch as a gene that encodes a chimeric HBc molecule does not occur naturally in plants, a contemplated transgenic plant accumulates chimeric HBc molecule particles in a greater amount than does a non-transformed plant of the same type or strain when both plants are grown under the same conditions.

The phrase "same type" or "same strain" is used herein to mean a plant of the same cross as or a clone of the untransformed plant. Where allelic variations among siblings of a cross are small, as with extensively inbred plant, comparisons between siblings can be used or an average arrived at using several siblings. Otherwise, clones are preferred for the comparison.

Seed from a transgenic plant is grown in the field greenhouse, window sill or the like, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for chimeric HBc molecule particle accumulation, preferably in the field, under a range of environmental conditions.

A transgenic plant homozygous for chimeric HBc molecule particle accumulation is crossed with a parent plant having other desired traits. The progeny, which are heterozygous or independently segregatable for chimeric HBc molecule particle accumulation, are backcrossed with one or the other parent to obtain transgenic plants that exhibit chimeric HBc molecule particle accumulation and the

other desired traits. The backcrossing of progeny with the parent may have to be repeated more than once to obtain a transgenic plant that possesses a number of desirable traits.

An insect cell system can also be used to express a HBc chimera. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) or baculovirus is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae.

The sequences encoding a chimera can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of chimera sequence renders the polyhedrin gene inactive and produces recombinant virus lacking coat protein. The recombinant viruses can then be used to infect, for example, *S. Frugiperda* cells or *Trichoplusia* larvae in which the HBc chimera can be expressed. E. Engelhard et al. (1994) *Proc. Natl. Acad. Sci., USA*, 91:3224-3227; and V. Luckow, *Insect Cell Expression Technology*, pp. 183-218, in Protein Engineering: Principles and Practice, J.L. Cleland et al. eds., Wiley-Liss, Inc, 1996). Heterologous genes placed under the control of the polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) are often expressed at high levels during the late stages of infection.

Recombinant baculoviruses containing the chimeric gene are constructed using the baculovirus shuttle vector system [Luckow et al. (1993) *J. Virol.*, 67:4566-4579], sold commercially as the Bac-To-Bac™ baculovirus expression system (Life Technologies). Stocks of recombinant viruses are

prepared and expression of the recombinant protein is monitored by standard protocols (O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, W.H. Freeman and Company, New York, 1992; and King et al., The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992).

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Alternatively, synthetic linkers containing one or more restriction endonuclease sites can be used to join the DNA segment to the expression vector, as noted before. The synthetic linkers are attached to blunt-ended DNA segments by incubating the blunt-ended DNA segments with a large excess of synthetic linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase.

Thus, the products of the reaction are DNA segments carrying synthetic linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction endonuclease and ligated into an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the synthetic linker. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including New England BioLabs, Beverly, MA. A

desired DNA segment can also be obtained using PCR technology in which the forward and reverse primers contain desired restriction sites that can be cut after amplification so that the gene can be inserted into the vector. Alternatively PCR products can be directly cloned into vectors containing T-overhangs (Promega Corp., A3600, Madison, WI) as is well known in the art.

The expressed chimeric protein self-assembles into particles within the host cells, whether in single cells or in cells within a multicelled host. The particle-containing cells are harvested using standard procedures, and the cells are lysed using a French pressure cell, lysozyme, sonicator, bead beater or a microfluidizer (Microfluidics International Corp., Newton MA). After clarification of the lysate, particles are precipitated with 45 percent ammonium sulfate, resuspended in 20 mM sodium phosphate, pH 6.8 and dialyzed against the same buffer. The dialyzed material is clarified by brief centrifugation and the supernatant subjected to gel filtration chromatography using Sepharose® CL-4B. Particle-containing fractions are identified, subjected to hydroxyapatite chromatography, and reprecipitated with ammonium sulfate prior to resuspension, dialysis and sterile filtration and storage at -70°C.

HBc Chimer Conjugates

Any hapten (immunogen) to which a B cell or T cell response is desired can be linked to a contemplated HBc chimer or chimer particle such as a chimer particle containing a heterologous linker residue such as a lysine, glutamic or aspartic acid,

cysteine or tyrosine in the loop region of Domain II and an added cysteine residue near the N-terminus in Domain I to form a HBc chimer conjugate. The hapten of interest typically is a B cell immunogen. The hapten can be a polypeptide, a protein, a carbohydrate (saccharide; i.e., oligo- or polysaccharide), or a non-polypeptide, non-carbohydrate chemical such as 2,4-dinitrobenzene or a medicament such as cocaine or nicotine. (it is thus seen that the word "hatpen" is used herein somewhat more broadly that is usual to include small molecules that do not themselves induce an immune response, as well as larger molecule such as proteins that often can themselves induce an immune response. A HBc chimer particle conjugate so formed is useful as an inoculum or vaccine, as is discussed hereinafter. Because the chimer protein self assembles upon expression and a conjugate is formed after expression, conjugate formation is typically done using the assembled particles as compared to the free protein molecules.

Methods for operatively linking individual haptens (immunogens) to a protein or polypeptide through an amino acid residue side chain of the protein or polypeptide to form a pendently-linked immunogenic conjugate, e.g., a branched-chain polypeptide polymer, are well known in the art. Those methods include linking through one or more types of functional groups on various side chains and result in the carrier protein polypeptide backbone (here, a HBc chimer) within the particle being pendently linked--covalently linked (coupled)-- to the hapten but separated by at least one side chain.

Methods for linking carrier proteins to haptens using each of the above functional groups are described in Erlanger, (1980) *Method of Enzymology*, 70:85; Aurameas et al., (1978) *Scand. J. Immunol.*, Vol. 8, Suppl. 7, 7-23 and U.S. Patent No. 4,493,795 to Nestor et al. In addition, a site-directed coupling reaction, as described in Rodwell et al. (1985) *Biotech.*, 3:889-894 can be carried out so that the biological activity of the polypeptides is not substantially diminished.

Furthermore, as is well known in the art, both the HBc protein and a polypeptide hapten can be used in their native form or their functional group content can be modified by succinylation of lysine residues or reaction with cysteine-thiolactone. A sulfhydryl group can also be incorporated into either carrier protein or conjugate by reaction of amino functional groups with 2-iminothiolane, the N-hydroxysuccinimide ester of 3-(3-dithiopyridyl)-propionate, or other reagents known in the art.

The HBc chimer or hapten can also be modified to incorporate a spacer arm, such as hexamethylenediamine or another bifunctional molecule, to facilitate the pendent linking. Such a procedure is discussed below.

Methods for covalent bonding of a polypeptide hapten are extremely varied and are well known by workers skilled in the immunological arts. For example, following U.S. Patent No. 4,818,527, m-maleimidobenzoyl-N-hydroxysuccinimide ester (ICN Biochemicals, Inc., Costa Mesa, CA) or succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce Chemical Co., Rockford, IL) is reacted with an appropriate HBc chimer to form an activated carrier.

That activated carrier is then reacted with a hapten such as a sulfhydryl-terminated hapten or a polypeptide that either contains a terminal cysteine or to which an additional amino- or carboxy-terminal cysteine residue has been added to form a covalently bonded HBC chimer conjugate. As an alternative example, the amino group of a polypeptide hapten can be first reacted with N-succinimidyl 3-(2-pyridylthio)propionate (SPDP, Pharmacia, Piscataway, NJ), and that thiol-containing polypeptide can be reacted with the activated carrier after reduction. Of course, the sulfur-containing moiety and double bond-containing Michael acceptor can be reversed. These reactions are described in the supplier's literature, and also in Kitagawa, et al. (1976) *J. Biochem.*, 79:233 and in Lachmann et al., in 1986 Synthetic Peptides as Antigens, (Ciba Foundation Symposium 119), pp. 25-40 (Wiley, Chichester: 1986).

U.S. Patent No. 4,767,842 teaches several modes of covalent attachment between a carrier and polypeptide that are useful here. In one method, tolylene diisocyanate is reacted with the carrier in a dioxane-buffer solvent at zero degrees C to form an activated carrier. A polypeptide hapten is thereafter admixed and reacted with the activated carrier to form the covalently bonded HBC chimer conjugate.

Particularly useful are a large number of heterobifunctional agents that form a disulfide link at one functional group end and an amide link at the other, including N-succidimidyl-3-(2-pyridyldithio)-propionate (SPDP), discussed before that creates a disulfide linkage between itself and a thiol in either the HBC chimer or the hapten. Exemplary

$$\frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} f(x) e^{-x^2} dx = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} f(x) e^{-x^2} dx = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} f(x) e^{-x^2} dx$$

- 87 -

Exemplary polypeptide haptens are shown in Tables A and B hereinbefore. Each of those polypeptides can be utilized via its N-terminal amino group, or by use of an additional N-terminal cysteine that is not shown in the table.

Related chemistry is used to couple what may be called "chemical compounds" to carrier proteins. Typically, an appropriate functional group for coupling is designed into the chemical compound. An exemplary chemical hapten to which induced antibodies protect against *Streptococcus pneumoniae* is 6-O-phosphocholine hydroxyhexanoate. Fischer et al. (1995) *J. Immunol.*, **154**:3373-3382. The table below provides further exemplary chemical haptens.

Chemical Haptens

Chemical Hapten	Citation
piperidine N-oxide	U.S. Patent No. 5,304,252
phospholactone or lactamide	U.S. Patent No. 5,248,611
metal ion complexes	U.S. Patent No. 5,236,825
[2.2.1] or [7.2.2] bicyclic ring compounds	U.S. Patent No. 5,208,152
ionically charged hydroxyl-containing compounds	U.S. Patent No. 5,187,086
phosphonate analogs of carboxylate esters	U.S. Patent No. 5,126,258
cocaine analogs	Carrera et al., (1995) <i>Nature</i> 378 :725

There are many methods known in the art to couple carrier proteins to polysaccharides. Aldehyde groups can be prepared on either the reducing end [Anderson (1983) *Infect. Immun.*, **39**:233-238; Jennings et al. (1981) *J. Immunol.*, **127**:1011-1018; Poren et al. (1985) *Mol. Immunol.*, **22**:907-919] or the terminal end [Anderson et al. (1986) *J. Immunol.*, **137**:1181-1186; Beuvery et al. (1986) *Dev. Bio. Scand.*, **65**:197-204] of an oligosaccharide or relatively small polysaccharide, which can be linked to the carrier protein via reductive amination.

Large polysaccharides can be conjugated by either terminal activation [Anderson et al. (1986) *J. Immunol.*, **137**:1181-1186] or by random activation of several functional groups along the polysaccharide chain [Chu et al. (1983) *Infect. Immun.*, **40**:245-256; Gordon, U.S. Patent No. 4,619,828 (1986); Marburg, U.S. Patent No. 4,882,317 (1989)]. Random activation of several functional groups along the polysaccharide chain can lead to a conjugate that is highly cross-linked due to random linkages along the polysaccharide chain. The optimal ratio of polysaccharide to carrier protein depends on the particular polysaccharide, the carrier protein, and the conjugate used.

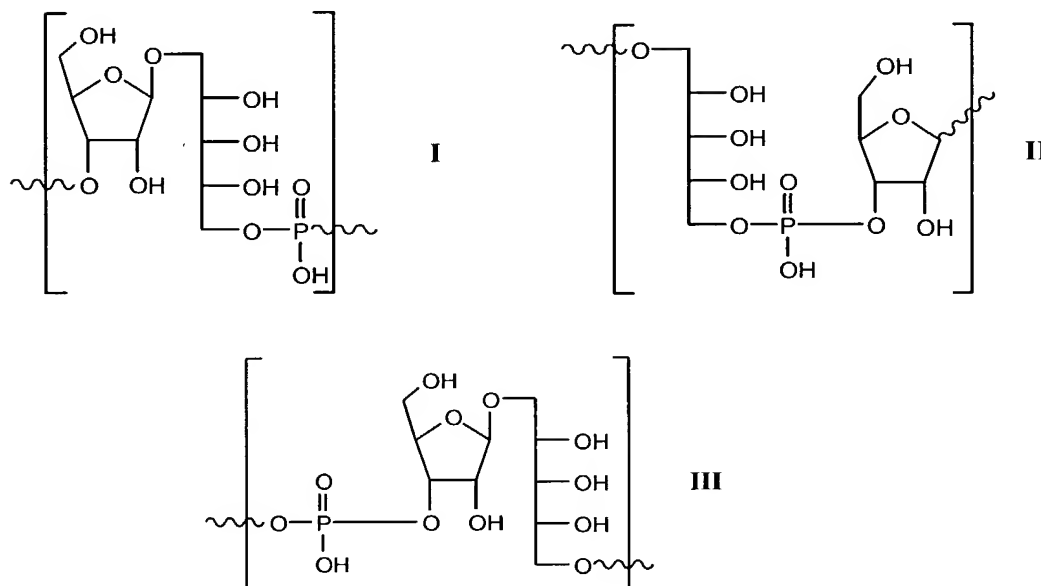
Detailed reviews of methods of conjugation of saccharide to carrier proteins can be found in Dick et al., in Contributions to Microbiology and Immunology, Vol. 10, Cruse et al., eds., (S. Karger: 1989), pp. 48-114; Jennings et al., in Neoglycoconjugates: Preparation and Applications, Lee et al., eds., (Academic Press: 1994), pp. 325-371; Aplin et al., (1981) *CRC Crit. Rev. Biochem.*,

10:259-306; and Stowell et al. (1980) *Adv. Carbohydr. Chem. Biochem.*, 37:225-281.

The carbohydrate itself can be synthesized by methods known in the art, for example by enzymatic glycoprotein synthesis as described by Witte et al. (1997) *J. Am. Chem. Soc.*, 119:2114-2118.

Several oligosaccharides, synthetic and semi-synthetic, and natural, are discussed in the following paragraphs as examples of oligosaccharides that are contemplated haptens to be used in making a HBc conjugate of the present invention.

An oligosaccharide hapten suitable for preparing vaccines for the treatment of *Haemophilus influenza* type b (Hib) is made up of from 2 to 20 repeats of D-ribose-D-ribitol-phosphate (I, below), D-ribitol-phosphate-D-ribose (II, below), or phosphate-D-ribose-D-ribitol (III, below). Eduard C. Beuvery et al., EP-0 276 516-B1.



U.S. Patent No. 4,220,717 also discloses a polyribosyl ribitol phosphate (PRP) hapten for *Haemophilus influenzae* type b.

Peterson et al. (1998) *Infect. Immun.*, 66(8):3848-3855, disclose a trisaccharide hapten, $\alpha\text{Kdo}(2 \rightarrow 8)\alpha\text{Kdo}(2 \rightarrow 4)\alpha\text{Kdo}$, that provides protection from *Chlamydia pneumoniae*. *Chlamydia pneumoniae* is a cause of human respiratory infections ranging from pharyngitis to fatal pneumonia. Kdo is 3-deoxy-D-manno-oct-2-ulosonic acid.

Andersson et al., EP-0 126 043-A1, disclose saccharides that can be used in the treatment, prophylaxis or diagnosis of bacterial infections caused by *Streptococci pneumoniae*. One class of useful saccharides is derived from the disaccharide $\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}$. Andersson et al. also reported neolactotetraosylceramide to be useful, which is $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc-Cer}$.

McKenney et al. (1999) *Science*, 284:1523-1527, disclose a polysaccharide, poly-N-succinyl $\beta 1 \rightarrow 6\text{GlcN}$ (PNSG) that provides protection from *Staphylococcus aureus*. *S. aureus* is a common cause of community-acquired infections, including endocarditis, osteomyelitis, septic arthritis, pneumonia, and abscesses.

European Patent No. 0 157 899-B1 discloses the isolation of pneumococcal polysaccharides that are useful in the present invention. The following table lists the pneumococcal culture types that produce capsular polysaccharides useful as haptens in the present invention.

Polysaccharide Hapten Sources

Danish Type Nomenclature	U.S. Nomenclature	1978 ATCC Catalogue Number
1	1	6301
2	2	6302
3	3	6303
4	4	6304
5	5	
6A	6	6306
6B	26	6326
7F	51	10351
8	8	6308
9N	9	6309
9V	68	
10A	34	
11A	43	
12F	12	6312
14	14	6314
15B	54	
17F	17	
18C	56	10356
19A	57	
19F	19	6319
20	20	6320
22F	22	
23F	23	6323
25	25	6325
33F	70	

Moraxella (Branhamella) catarrhalis is a reported cause of otitis media and sinusitis in children and lower respiratory tract infections in adults. The lipid A portion of the lipooligosaccharide surface antigen (LOS) of the bacterium is cleaved at the 3-deoxy-D-manno-octulosonic acid-

glucosamine linkage. The cleavage product is treated with mild-alkali to remove ester-linked fatty acids, while preserving amide-linked fatty acids to yield detoxified lipopolysaccharide (dLOS) from *M. catarrhalis*. The dLOS is not immunogenic until it is attached to a protein carrier. Xin-Xing Gu et al. (1998) *Infect. Immun.*, **66**(5):1891-1897.

Group B streptococci (GBS) is a cause of sepsis, meningitis, and related neurologic disorders in humans. The Capsular polysaccharide-specific antibodies are known to protect human infants from infection. Jennings et al., U.S. Patent No. 5,795,580. The repeating unit of the GBS capsular polysaccharide type II is: $4)-\beta\text{-D-GlcpNAc-(1 3)-}[\beta\text{-D-Galp(1 6)}]-\beta\text{-D-Galp(1 4)-}\beta\text{-D-Glcp-(1 3)-}\beta\text{-D-Glcp-(1 2)-}[\alpha\text{-D-NeupNAc(2 3)}]-\beta\text{-D-Galp-(1$, where the bracketed portion is a branch connected to the immediately following unbracketed subunit. The repeating unit of GBS capsular polysaccharide type V is: $4)-[\alpha\text{-D-NeupNAc-(2 3)-}\beta\text{-D-Galp-(1 4)-}\beta\text{-D-GlcpNAc-(1 6)}]-\alpha\text{-D-Glcp-(1 4)-}[\beta\text{-D-Glcp-(1 3)}]-\beta\text{-D-Galp-(1 4)-}\beta\text{-D-Glcp-(1$.

European patent application No. EU-0 641 568-A1, Brade, discloses the method of obtaining ladder-like banding pattern antigen from *Chlamydia trachomatis*, *pneumoniae* and *psittaci*.

Slovin et al., (1999) *Proc. Natl. Acad. Sci., U.S.A.*, **96**(10):5710-5715 report use of a synthetic oligosaccharide, globo H, linked to KLH as a carrier in the preparation of a vaccine used against prostate cancer. Similarly, Helling et al., (July 1995) *Cancer Res.*, **55**:2783-2788 report the use of KLH-linked G_{M2} in a vaccine for treating patients

with melanoma. The latter vaccine was prepared by ozone cleavage of the ceramide double bond of G_{M2} , introduction of an aldehyde group and reductive alkylation onto KLH. A similar procedure can be utilized with a contemplated chimer particle.

Oligosaccharidal portions of sphingolipids such as globosides and gangliosides that are present on the surface of other tumor cells as well as normal cells such as melanoma, neuroblastoma and healthy brain cells can similarly be used herein as a hapten. The oligosaccharide portion of the globoside globo H has the structure $Fuc\alpha-(1\ 2)-Gal\beta(1\ 3)-GalNAc\beta-(1\ 3)-Gal\alpha-(1\ 4)-Gal\beta-(1\ 4)Glc$, whereas the saccharide portions of gangliosides G_{M2} , G_{M1} and G_{D1a} have the following structures: $GalNAc\beta-(1\ 4)-[NeuAc\alpha-(2\ 3)]-Gal\beta-(1\ 4)-Glc$; $Gal\beta-(1\ 3)-GalNAc\beta-(1\ 4)-[NeuAc\alpha-(2\ 3)]-Gal\beta-(1\ 4)-Glc$; and $NeuAc-(2\ 3)-Gal\beta-(1\ 3)-GalNAc\beta-(1\ 4)-[NeuAc\alpha-(2\ 3)]-Gal\beta-(1\ 4)-Glc$, respectively.

U.S. Patent No. 4,356,170 discloses the preparation of useful polysaccharides that are reduced and then oxidized to form compounds having terminal aldehyde groups that can be reductively aminated onto free amine groups of carrier proteins such as tetanus toxoid and diphtheria toxoid with or without significant cross-linking. Exemplary useful bacterial polysaccharides include β -hemolytic streptococci, *Haemophilus influenza*, meningococci, pneumococci and *E. coli*. Rather than reductively aminating the particles, a linker arm such as that provided by an ϵ -amino C_2-C_8 alkylcarboxylic acid can be reductively aminated on to the polysaccharide,

followed by linkage to the particles using a water-soluble carbodiimide.

Inocula and Vaccines

In yet another embodiment of the invention, a HBc chimer particle or HBc chimer particle conjugate with a hapten is used as the immunogen of an inoculum that induces a B cell or T cell response (stimulation) in an inoculated host animal such as production of antibodies that immunoreact with the heterologous epitope or hapten or T cell activation, or as a vaccine to provide protection against the pathogen, protein or other entity from which the heterologous epitope or the hapten is derived.

T cell activation can be measured by a variety of techniques. In usual practice, a host animal is inoculated with a contemplated HBc chimer particle vaccine or inoculum, and peripheral mononuclear blood cells (PMBC) are thereafter collected. Those PMBC are then cultured *in vitro* in the presence of the T cell immunogen for a period of about three to five days. The cultured PMBC are then assayed for proliferation or secretion of a cytokine such as IL-2, GM-CSF or IFN- γ . Assays for T cell activation are well known in the art. See, for example, U. S. Patent No. 5,478,726 and the art cited therein.

Using antibody formation as exemplary, a contemplated inoculum or vaccine comprises an immunogenic effective amount of HBc chimer particles or HBc chimer particle conjugates that are dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When administered to a host animal in need of immunization

or in which antibodies are desired to be induced such as a mammal (e.g., a mouse, dog, goat, sheep, horse, bovine, monkey, ape, or human) or bird (e.g., a chicken, turkey, duck or goose), an inoculum induces antibodies that immunoreact with the conjugated (pendently-linked) hapten. Those antibodies also preferably bind to the protein or saccharide of the B cell immunogen.

A vaccine is a type of inoculum in which the heterologous B cell epitope or conjugated hapten corresponds to a portion of a protein or saccharidal structure that is related to a disease state, as is an exemplary malarial B cell sequence related to a malarial pathogen. The vaccine-induced antibodies not only immunoreact with the epitope or hapten or activated T cells respond to that heterologous epitope or hapten, but also immunoreact with the pathogen or diseased cell *in vivo*, and provide protection from that disease state.

The amount of recombinant HBc chimer immunogen utilized in each immunization is referred to as an immunogenic effective amount and can vary widely, depending *inter alia*, upon the recombinant HBc chimer immunogen, mammal immunized, and the presence of an adjuvant in the vaccine, as discussed below. Immunogenic effective amounts for a vaccine and an inoculum provide the protection or antibody activity, respectively, discussed hereinbefore.

Vaccines or inocula typically contain a recombinant HBc chimer immunogen concentration of about 1 microgram to about 1 milligram per inoculation (unit dose), and preferably about 10 micrograms to about 50 micrograms per unit dose. The term "unit dose" as it pertains to a vaccine or inoculum of the present invention

[illegible]

...the ... of ...

The immunogenic active ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, an inoculum or vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying

agents, pH buffering agents that enhance the immunogenic effectiveness of the composition.

A contemplated vaccine or inoculum advantageously also includes an adjuvant. Suitable adjuvants for vaccines and inocula of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against B cell epitopes of the chimer, as well as adjuvants capable of enhancing cell mediated responses towards T cell epitopes contained in the chimer. Adjuvants are well known in the art (see, for example, Vaccine Design - The Subunit and Adjuvant Approach, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X).

Exemplary adjuvants include complete Freund's adjuvant (CFA) that is not used in humans, incomplete Freund's adjuvant (IFA), squalene, squalane and alum [e.g., Alhydrogel™ (Superfos, Denmark)], which are materials well known in the art, and are available commercially from several sources.

Preferred adjuvants for use with immunogens of the present invention include aluminum or calcium salts (for example hydroxide or phosphate salts). A particularly preferred adjuvant for use herein is an aluminum hydroxide gel such as Alhydrogel™. For aluminum hydroxide gels, the chimer protein is admixed with the adjuvant so that between 50 to 800 micrograms of aluminum are present per dose, and preferably between 400 and 600 micrograms are present.

Another particularly preferred adjuvant for use with an immunogen of the present invention is an emulsion. A contemplated emulsion can be an oil-in-

water emulsion or a water-in-oil emulsions. In addition to the immunogenic chimer protein, such emulsions comprise an oil phase of squalene, squalane, peanut oil or the like as are well-known, and a dispersing agent. Non-ionic dispersing agents are preferred and such materials include mono- and di-C₁₂-C₂₄-fatty acid esters of sorbitan and mannide such as sorbitan mono-stearate, sorbitan mono-oleate and mannide mono-oleate. An immunogen-containing emulsion is administered as an emulsion.

Preferably, such emulsions are water-in-oil emulsions that comprise squalene and mannide mono-oleate (ArlacelTM A), optionally with squalane, emulsified with the chimer protein in an aqueous phase. Well-known examples of such emulsions include MontanideTM ISA-720, and MontanideTM ISA 703 (Seppic, Castres, France), each of which is understood to contain both squalene and squalane, with squalene predominating in each, but to a lesser extent in MontanideTM ISA 703. Most preferably, MontanideTM ISA-720 is used, and a ratio of oil-to-water of 7:3 (w/w) is used. Other preferred oil-in-water emulsion adjuvants include those disclosed in WO 95/17210 and EP 0 399 843.

The use of small molecule adjuvants is also contemplated herein. One type of small molecule adjuvant useful herein is a 7-substituted-8-oxo- or 8-sulfo-guanosine derivative described in U.S. Patents No. 4,539,205, No. 4,643,992, No. 5,011,828 and No. 5,093,318, whose disclosures are incorporated by reference. Of these materials, 7-allyl-8-oxoguanosine (loxoribine) is particularly preferred. That molecule has been shown to be particularly

effective in inducing an antigen-(immunogen-)specific response.

Still further useful adjuvants include monophosphoryl lipid A (MPL) available from Corixa Corp. (see, U.S. Patent No. 4,987,237), CPG available from Coley Pharmaceutical Group, QS21 available from Aquila Biopharmaceuticals, Inc., SBAS2 available from SKB, the so-called muramyl dipeptide analogues described in U.S. Patent No. 4,767,842, and MF59 available from Chiron Corp. (see, U.S. Patents No. 5,709,879 and No. 6,086,901).

More particularly, immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree *Quillaja Saponaria Molina* (e.g. Quil™ A) are also useful. Derivatives of Quil™ A, for example QS21 (an HPLC purified fraction derivative of Quil™ A), and the method of its production is disclosed in U.S. Patent No. 5,057,540. In addition to QS21 (known as QA21), other fractions such as QA17 are also disclosed.

3-De-O-acylated monophosphoryl lipid A is a well-known adjuvant manufactured by Ribi Immunochem, Hamilton, Montana. The adjuvant contains three components extracted from bacteria, monophosphoryl lipid (MPL) A, trehalose dimycolate (TDM) and cell wall skeleton (CWS) (MPL+TDM+CWS) in a 2% squalene/Tween® 80 emulsion. This adjuvant can be prepared by the methods taught in GB 2122204B. A preferred form of 3-de-O-acylated monophosphoryl lipid A is in the form of an emulsion having a small particle size less than 0.2 µm in diameter (EP 0 689 454 B1).

The muramyl dipeptide adjuvants include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thur-MDP),

N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamin (CGP) 1983A, referred to as MTP-PE).

Preferred adjuvant mixtures include combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil-in-water emulsions comprising 3D-MPL and QS21 (WO 95/17210, PCT/EP98/05714), 3D-MPL formulated with other carriers (EP 0 689 454 B1), QS21 formulated in cholesterol-containing liposomes (WO 96/33739), or immunostimulatory oligonucleotides (WO 96/02555). Alternative adjuvants include those described in WO 99/52549 and non-particulate suspensions of polyoxyethylene ether (UK Patent Application No. 9807805.8).

Adjuvants are utilized in an adjuvant amount, which can vary with the adjuvant, mammal and recombinant HBc chimer immunogen. Typical amounts can vary from about 1 µg to about 1 mg per immunization. Those skilled in the art know that appropriate concentrations or amounts can be readily determined.

Inocula and vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations that are suitable for other modes of administration include suppositories and, in some cases, oral formulation. The use of a nasal spray for inoculation is also contemplated as discussed in Neirynck et al. (Oct. 1999) *Nature Med.*, 5(10):1157-1163. For suppositories, traditional binders and carriers can include, for example, polyalkalene glycols or triglycerides; such suppositories may be

formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like.

An inoculum or vaccine composition takes the form of a solution, suspension, tablet, pill, capsule, sustained release formulation or powder, and contains an immunogenic effective amount of HBc chimer or HBc chimer conjugate, preferably as particles, as active ingredient. In a typical composition, an immunogenic effective amount of preferred HBc chimer or HBc chimer conjugate particles is about 1 μ g to about 1 mg of active ingredient per dose, and more preferably about 5 μ g to about 50 μ g per dose, as noted before.

A vaccine is typically formulated for parenteral administration. Exemplary immunizations are carried out sub-cutaneously (SC) intra-muscularly (IM), intravenously (IV), intraperitoneally (IP) or intra-dermally (ID). However, oral and nasal routes of vaccination are also contemplated.

The HBc chimer particles and HBc chimer particle conjugates can be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein or hapten) and are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic

bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

In yet another embodiment, a vaccine or inoculum is contemplated in which a gene encoding a contemplated HBc chimera is transfected into suitably attenuated enteric bacteria such as *S. typhi*, *S. typhimurium*, *S. typhimurium-E. coli* hybrids or *E. coli*. Exemplary attenuated or avirulent *S. typhi* and *S. typhimurium* and *S. typhimurium-E. coli* hybrids are discussed in the citations provided before. These vaccines and inocula are particularly contemplated for use against diseases that infect or are transmitted via mucosa of the nose, the gut and reproductive tract such as influenza, yeasts such as *Aspergillus* and *Candida*, viruses such as polio, mumps and mouth disease, hepatitis A, and bacteria such as *Cholera*, *Salmonella* and *E. coli* and where a mucosal IgA response is desired in addition to or instead of an IgG systemic response.

The enteric bacteria can be freeze dried, mixed with dry pharmaceutically acceptable diluents, made into tablets or capsules for ingestion and administered to or taken by the host animal as are usual solid phase medications. In addition, aqueous preparations of these bacterial vaccines are adapted for use in mucosal immunization as by oral, nasal, rectal or vaginal administration.

Oral immunization using plant matter containing contemplated chimeric molecule particles can be achieved by simple ingestion of the transgenic plant tissue such as a root like a carrot or seed

such as rice or corn. In this case, the water of the mouth or gastrointestinal tract provides the usually used aqueous medium used for immunization and the surrounding plant tissue provides the pharmaceutically acceptable diluent.

The inocula or vaccines are administered in a manner compatible with the dosage formulation, and in such amount as are therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of tens of micrograms active ingredient per individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in intervals (weeks or months) by a subsequent injection or other administration.

Once immunized, the mammal is maintained for a period of time sufficient for the recombinant HBc chimera immunogen to induce the production of a sufficient titer of antibodies that bind to an antigen of interest such as a sporozoite for a malarial vaccine. The maintenance time for the production of illustrative anti-sporozoite antibodies typically lasts for a period of about three to about twelve weeks, and can include a booster, second immunizing administration of the vaccine. A third immunization is also contemplated, if desired, at a time 24 weeks to five years after the first

immunization. It is particularly contemplated that once a protective level titer of antibodies is attained, the vaccinated mammal is preferably maintained at or near that antibody titer by periodic booster immunizations administered at intervals of about 1 to about 5 years.

The production of anti-sporozoite or other antibodies is readily ascertained by obtaining a plasma or serum sample from the immunized mammal and assaying the antibodies therein for their ability to bind to an appropriate antigen such as a synthetic circumsporozoite immunodominant antigen [e.g. the *P. falciparum* CS protein peptide (NANP)₅ used herein] in an ELISA assay as described hereinafter or by another immunoassay such as a Western blot as is well known in the art.

It is noted that the induced antibodies such as anti-CS antibodies or anti-influenza antibodies can be isolated from the blood of an inoculated host mammal using well known techniques, and then reconstituted into a second vaccine for passive immunization as is also well known. Similar techniques are used for gamma-globulin immunizations of humans. For example, antiserum from one or a number of immunized hosts can be precipitated in aqueous ammonium sulfate (typically at 40-50 percent of saturation), and the precipitated antibodies purified chromatographically as by use of affinity chromatography in which (NANP)₅ or an influenza M2 polypeptide is utilized as the antigen immobilized on the chromatographic column. Thus, for example, an inoculum can be used in a horse or sheep to induce antibody production against a malarial species for

use in a passive immunization in yet another animal such as humans.

Another embodiment of the invention is a process for inducing antibodies, activated T cells or both in an animal host comprising the steps of inoculating said animal host with an inoculum. The inoculum used in the process comprises an immunogenic amount of a before-described HBc chimer particle or HBc chimer particle conjugate dissolved or dispersed in a pharmaceutically acceptable diluent. The animal host is maintained for a time sufficient for antibodies or activated T cells to be induced, as can be assayed by well-known techniques, which typically requires a time period of weeks to months, as is again well-known. A plurality of such immunizations is contemplated during this maintenance period.

The invention is illustrated by the following non-limiting examples.

Example 1: B Cell Epitope-Containing Chimer Preparation

A. Preparation of plasmid vector pKK223- 3N, a modified form of pKK223-3

Plasmid vector pKK223-3 (Pharmacia) was modified by the establishment of a unique NcoI restriction site to enable insertion of HBc genes as NcoI-HindIII restriction fragments and subsequent expression in *E.coli* host cells. To modify the pKK223-3 plasmid vector, a new SphI-HindIII fragment was prepared using the PCR primers pKK223-3/433-452-F and pKK223-NcoI-mod-R, and pKK223-3 as the template. This PCR fragment was cut with the restriction enzymes SphI and HindIII to provide a 467 bp fragment

that was then ligated with a 4106 bp fragment of the pKK223-3 vector, to effectively replace the original 480 bp SphI-HindIII fragment. The resultant plasmid (pKK223-3N) is therefore 13 bp shorter than the parent plasmid and contains modified nucleotide sequence upstream of the introduced NcoI site (see Fig. 1 in which the dashes indicate the absent bases). The final plasmid, pKK223-3N, has a size of 4573 bp. Restriction sites in plasmid pKK223-3N are indicated in Fig. 1, and the nucleotide changes made to pKK223-3 to form plasmid pKK223-3N are indicated by an underline as shown below.

pKK223-3/433-452-F GGTGCATGCAAGGAGATG SEQ ID NO:178

pKK223-NcoI-mod-R

GCGAAGCTTCGGATCccatggTTTTTTCCTCCTTATGTGAAATTGTTATCCG-
CTC SEQ ID NO:179

B. Preparation of V1 and V2 Cloning Vectors

Modified HBc149 genes, able to accept the directional insertion of synthetic dsDNA fragments into the immunodominant loop region, were constructed using PCR. [The plasmid accepting inserts between amino acids E77 and D78 was named V1, whereas the plasmid accepting inserts between D78 and P79 was named V2.] The HBc149 gene was amplified in two halves using two PCR primer pairs, one of which amplifies the amino terminus, the other amplifies the carboxyl terminus. For V1, the products of the PCR reactions (N- and C-terminus) are both 246 bp fragments; for V2, the products are a 249 bp (N-terminus) and a 243 bp fragment (C-terminus).

The N-terminal fragments prepared were digested with NcoI and EcoRI, and the C-terminal fragments were digested with EcoRI and HindIII. The V1 and V2 fragments pairs were then ligated together at the common EcoRI overhangs. The resultant NcoI-HindIII fragments were then ligated into the pKK223-3N vector, which had been prepared by digestion with NcoI and HindIII.

To insert B cell epitopes into the V1 and V2 plasmids, the plasmids were digested with EcoRI and SacI restriction enzymes. Synthetic dsDNA fragments containing 5' EcoRI and 3' SacI overhangs were then inserted. In both cases, V1 and V2, glycine-isoleucine (EcoRI) and glutamic acid-leucine (SacI) amino acid pairs, coded for by the restriction sites, flank the inserted B cell epitopes. The inserted restriction sites are underlined in the primers below.

V1 _____

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA SEQ ID NO:180

5'-GGTGGCTTACCAGAGGCCGGAGGC

AATGGATCATTCTGAACCG SEQ ID NO:290

HBc-E77/EcoRI-R

5'-GCGGAATTCCTTCCAAATTAACACCCACC SEQ ID NO:181

HBc-D78/EcoRI-SacI-F

5'-CGCGAATTCAAAAAGAGCTCGATCCAGCGTCTAGAGAC

SEQ ID NO:182

HBc149/HindIII-R

5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG

SEQ ID NO:183

V2

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA

SEQ ID NO:180

HBc-D78/EcoRI-R

5'-GCGGAATTCCATCTTCCAAATTAACACCCAC

SEQ ID NO:184

HBc-P79/EcoRI-SacI-F

5'-CGCGAATTCAAAAAGAGCTCCCAGCGTCTAGAGACCTAG

SEQ ID NO:185

HBc149/HindIII-R

5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG

SEQ ID NO:183

Vectors to Express Chimer Particles Containing an N-Terminal Cysteine and the CS-Repeat Epitopes from *P.falciparum* in the Immunodominant Loop

Two expression vectors [V2.Pf1(N-MGCELDP) and V2.Pf1(N-MGCDIDP)] are prepared to determine the ability of N-terminal cysteine residues to stabilize chimer particles. To make the vector V2.Pf1(N-MGCELDP), the oligonucleotides HBc(MGCELDP)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 528 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which had been cleaved with the same two enzymes.

To make the vector V2.Pf1(N-MGCDIDP) the oligonucleotides HBc(MGCDIDP)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 528 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which has been cleaved with the same two enzymes.

HBc (MGCELDP) -NcoI-F

M G C E L D P Y K E F G SEQ ID NO:186
5'-GCGCCATGGGGTGTGAGCTCGACCCTTATAAAGAATTGG SEQ ID NO:187

HBc (MGCDIDP) -NcoI-F

M G C D I D P Y K E F G SEQ ID NO:188
5'-GCGCCATGGGGTGTGACATCGACCCTTATAAAGAATTGG SEQ ID NO:189

C. Preparation of V7 Cloning Vector

To enable the fusion of T cell epitopes to the C terminus of a HBc chimera, a new vector, V7, was constructed. Unique EcoRI and SacI restriction sites were inserted between valine-149 and the HindIII site to facilitate directional insertion of synthetic dsDNAs into EcoRI-HindIII (or EcoRI-SacI) restriction sites. The pair of PCR primers below was used to amplify the HBc 149 gene with a NcoI restriction site at the amino-terminus and EcoRI, SacI and HindIII sites at the carboxyl-terminus. The product of the PCR reaction (479 bp) was digested with NcoI/HindIII and cloned into pKK223-3N to form V7.

To insert T cell epitopes, the plasmid (V7) was digested EcoRI/HindIII (or EcoRI-SacI) and synthetic dsDNA fragments having EcoRI/HindIII (or

EcoRI/SacI) overhangs, were ligated into V7. For all V7 constructs, the final amino acid of native HBc (valine-149) and the first amino acid of the inserted T cell epitope are separated by a glycine-isoleucine dipeptide sequence coded for by the nucleotides that form the EcoRI restriction site. For epitopes inserted at EcoRI/SacI, there are additional glutamic acid-leucine residues after the T cell epitope, prior to the termination codon, contributed by the SacI site. Restriction sites are again underlined in the primers shown.

HBc149/NcoI-F

5'-TTGGGCCATGGACATCGACCCTTA

SEQ ID NO:180

HBc149/SacI-EcoRI-H3-R

5'-CGCAAGCTTAGAGCTCTTGAATTCCAACAACAGTAGTCTCCG

SEQ ID NO:190

D. Preparation of V12

Expression Constructs

V12 vectors, which contain B cell epitopes between amino acids 78 and 79, as well as T cell epitopes downstream of valine-149, are constructed from V2 and V7 vectors. The carboxyl terminus of a V7 vector containing a T cell epitope inserted at EcoRI/HindIII is amplified using two PCR primers (HBc-P79/SacI-F and pKK223-2/4515-32R) to provide a dsDNA fragment corresponding to amino acids 79-149 plus the T cell epitope, flanked with SacI and HindIII restriction sites.

The PCR products are cut with SacI and HindIII and then cloned into the desired V2 vector prepared by cutting with the same two enzymes. The

PCR primers are amenable for the amplification of the carboxyl terminus of all V7 genes, irrespective of the T cell epitope present after amino acid 149 of the HBc gene.

One exception to the generality of this approach was in the preparation of the V12 constructs containing the Pf-CS(C17A) mutation, which were prepared from existing V12 constructs. In this case, V12 constructs were amplified with HBc149/NcoI-F (SEQ ID NO:180) and the mis-match reverse PCR primer (SEQ ID NO:292), which facilitated the C17A mutation. The resultant PCR product was digested with NcoI and HindIII and cloned back into pKK223-3N (previously cut with the same enzymes). Restriction sites are underlined.

HBc-P79/SacI-F 5'-CGCGGAGCTCCCAGCGTCTAGAGACCTAG
SEQ ID NO:191

pKK223-2/4515-32R 5'-GTATCAGGCTGAAAATC
SEQ ID NO:192

E. *P.falciparum* CS-repeat B cell

Epitopes Inserted into V2

For V2 and V7 constructs, synthetic dsDNA fragments coding for the B (V2) or T cell epitope (V7) of interest are inserted into EcoRI/SacI restriction sites. Synthetic dsDNA fragments, encoding B and T cell epitopes of interest, are prepared by mixing complementary single stranded DNA oligonucleotides at equimolar concentrations, heating to 95°C for 5 minutes, and then cooling to room temperature at a rate of -1 °C per minute. This annealing reaction is performed in TE buffer. The

double-stranded DNAs are shown below with the encoded epitope sequence shown above. The pound symbol, #, is used in some of the amino acid residue sequences that follow to indicate the presence of a stop codon.

Pf1

I N A N P N A N P N A N P N A
AATTAACGCTAATCCGAACGCTAATCCGAACGCTAATCCGAACGCTA
TTGCGATTAGGCTTGCGATTAGGCTTGCGATTAGGCTTGCGAT

N P E L	SEQ ID NO:193
ATCCGGAGCT	SEQ ID NO:194
TAGGCC	SEQ ID NO:195

Pf3

I N A N P N V D P N A N P N A N P
AATTAACGCTAATCCGAACGTTGACCCGAACGCTAATCCGAACGCTAATCCGA
TTGCGATTAGGCTTGCAACTGGGCTTGCGATTAGGCTTGCGATTAGGCT

N A N P N V D P N A N P E L SEQ ID NO:196
ACGCTAATCCGAACGTTGACCCGAACGCTAATCCGGAGCT SEQ ID NO:197
TGCGATTAGGCTTGCAACTGGGCTTGCGATTAGGCCTCGAGG
SEQ ID NO:198

Pf3.1

I N A N P N V D P N A N P N A N P
AATTAACGCGAATCCGAACGTGGATCCGAATGCCAACCTAACGCCAACCC
TTGCGCTTAGGCTTGACCTAGGCTTACGGTTGGGATTGCGGTTGGG

N A N P E L
AAATGCGAACCCAGAGCT
TTTACGCTTGGGTC

SEQ ID NO:199
SEQ ID NO:200
SEQ ID NO:201

Pf3.2

I N A N P N A N P N A N P N V D P
AATTAACGCGAATCCGAATGCCAACCCCTAACGCCAACCCAAACGTGGATCCGA
TTGCGCTTAGGCTTACGGTTGGGATTGCGGTTGGGTTTGCACCTAGGCT

N A N P E L
ATGCGAACCCAGAGCT
TACGCTTGGGTC

SEQ ID NO:202
SEQ ID NO:203
SEQ ID NO:204

Pf3.3

I N A N P N V D P N A N P N A N P
AATTAACGCGAATCCGAACGTGGATCCAAATGCCAACCCCTAACGCTAATCCAA
TTGCGCTTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTT

N A N P N V D P N A N P E L SEQ ID NO:205
ACGCCAACCCGAATGTTGACCCCAATGCCAATCCGGAGCT SEQ ID NO:206
TGCGGTTGGGCTTACAACCTGGGGTTACGGTTAGGCC SEQ ID NO:207

Pf3.4

I N P N V D P N A N P N A N P N A
AATTAATCCGAACGTGGATCCAAATGCCAACCCCTAACGCTAATCCAAACGCCA
TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT

N P N V E L

SEQ ID NO:208

ACCCGAATGTTGAGCT

SEQ ID NO:209

TGGGCTTACAAC

SEQ ID NO:210

Pf3.5

I N P N V D P N A N P N A N P N A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT

N P N V D P E L

SEQ ID NO:211

ACCCGAATGTTGACCCTGAGCT

SEQ ID NO:212

TGGGCTTACAACCTGGGAC

SEQ ID NO:213

Pf3.6

I N P N V D P N A N P N A N P N A
AATTAATCCGAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCA
TTAGGCTTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGT

N P N V D P N A E L

SEQ ID NO:214

ACCCGAATGTTGACCCTAATGCTGAGCT

SEQ ID NO:215

TGGGCTTACAACCTGGGATTACGAC

SEQ ID NO:216

Pf3.7

I N V D P N A N P N A N P N A N P
AATTAACGTGGATCCAAATGCCAACCCTAACGCTAATCCAAACGCCAACCCGA
TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT

SEQ ID NO:217

SEQ ID NO:218

SEQ ID NO:219

I N V D P N A N P N A N P N A N P

AATTAACGTGGATCCAAATGCCAACCCCTAACGCTAATCCAAACGCCAACCCGA

TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT

SEQ ID NO:220

SEQ ID NO:221

SEQ ID NO:222

I N V D P N A N P N A N P N A N P
AATTAACGTGGATCCAAATGCCAACCTAACGCTAATCCAAACGCCAACCCGA
TTGCACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGCGGTTGGGCT

SEQ ID NO:223

SEQ ID NO:224

SEQ ID NO:225

I D P N A N P N A N P N A N P
AATTGATCCAAATGCCAACCTAACGCTAATCCAAACGCCAACCTAGGTTTACGGTTGGGATTGCGATTAGGTTTGC GGTTGG

SEQ ID NO:226

SEQ ID NO:227

GCTTACAAC

SEQ ID NO:228

Pf3.11

I D P N A N P N A N P N A N P N V
AATTGATCCAAATGCCAACCCCTAACGCTAATCCAAACGCCAACCCGAATGTTG
CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGC GGTTGGGCTTACAAC

D P E L

SEQ ID NO:229

ACCCTGAGCT

SEQ ID NO:230

TGGGAC

SEQ ID NO:231

Pf3.12

I D P N A N P N A N P N A N P N V
AATTGATCCAAATGCCAACCCCTAACGCTAATCCAAACGCCAACCCGAATGTTG
CTAGGTTTACGGTTGGGATTGCGATTAGGTTTGC GGTTGGGCTTACAAC

D P N A E L

SEQ ID NO:232

ACCCTAATGCCGAGCT

SEQ ID NO:233

TGGGATTACGGC

SEQ ID NO:234

F. *P.falciparum* universal T cell epitope

Pf-UTC (PF/CS326-345)

I E Y L N K I Q N S L S T E W S P
AATTGAATATCTGAACAAAATCCAGAACTCTCTGTCCACCGAATGGTCTCCGT
CTTATAGACTTGTTTTAGGTCTTGAGAGACAGGTGGCTTACCAGAGGCA

C S V T # #

SEQ ID NO:235

GCTCCGTTACCTAGTA

SEQ ID NO:236

CGAGGCAATGGATCATTCGA

SEQ ID NO:237

P.vivax CS-repeat B cell epitopes

Pv-T1A

I P A G D R A D G Q P A G D R A A
AATTCCGGCTGGTGACCGTGCAGATGGCCAGCCAGCGGGTGACCGCGCTGCAG
GGCCGACCACTGGCACGTCTACCGGTCGGTCGCCCACTGGCGCGACGTC

G Q P A G E L

SEQ ID NO:238

GCCAGCCGGCTGGCGAGCT

SEQ ID NO:239

CGGTCGGCCGACCGC

SEQ ID NO:240

Pv-T1B

I D R A A G Q P A G D R A D G Q P
AATTGACAGAGCAGCCGGACAACCAGCAGGCGATCGAGCAGACGGACAGCCCG
CTGTCTCGTCGGCCTGTTGGTCGTCCGCTAGCTCGTCTGCCTGTCGGGC

A G E L

SEQ ID NO:241

CAGGGGAGCT

SEQ ID NO:242

GTCCCC

SEQ ID NO:243

Pv-T2A

I A N G A G N Q P G A N G A G D Q
AATTGCGAACGGCGCCGGTAATCAGCCGGGGGCAAACGGCGCGGGTGATCAAC
CGCTTGCCGCGGCCATTAGTCGGCCCCCGTTTGCCGCGCCCACTAGTTG

P G E L
CAGGGGAGCT
GTCCCC

SEQ ID NO:244
SEQ ID NO:245
SEQ ID NO:246

Pv-T2B

I A N G A D N Q P G A N G A D D Q
AATTGCGAACGGCGCCGATAATCAGCCGGGTGCAAACGGGGCGGATGACCAAC
CGCTTGCCGCGGCTATTAGTCGGCCACGTTTGCCCCGCTACTGGTTG

P G E L
CAGGCGAGCT
GTCCGC

SEQ ID NO:247
SEQ ID NO:248
SEQ ID NO:249

Pv-T2C

I A N G A G N Q P G A N G A G D Q
AATTGCGAACGGCGCCGGTAATCAGCCGGGAGCAAACGGCGCGGGGGATCAAC
CGCTTGCCGCGGCCATTAGTCGGCCCTCGTTTGCCGCGCCCCCTAGTTG

P G A N G A D N Q P G A N G A D D
CAGGCGCCAATGGTGCAGACAACCAGCCTGGGGCGAATGGAGCCGATGACC
GTCCGCGGTTACCACGTCTGTTGGTTCGGACCCCGCTTACCTCGGCTACTGG

Q P G E L
AACCCGGCGAGCT
TTGGGCCGC

SEQ ID NO:250
SEQ ID NO:251
SEQ ID NO:252

PV-T3

I A P G A N Q E G G A A A P G A N
AATTGCGCCGGGCGCCAACCAGGAAGGTGGGGCTGCAGCGCCAGGAGCCAATC

CGCGGCCCGCGGTTGGTCCTTCCACCCCGACGTCGCGGTCCTCGGTTAG

Q E G G A A E L	SEQ ID NO:253
AAGAAGGCGGTGCAGCGGAGCT	SEQ ID NO:254
TTCTTCCGCCACGTCGCC	SEQ ID NO:255

Example 2: Assay Procedures

A. Antigenicity

1. Particle ELISA

Purified particles were diluted to a concentration of 10 µg/mL in coating buffer (50 mM sodium bicarbonate, pH 9.6) and coated onto the wells of ELISA strips (50 µL/well). The ELISA strips are incubated at room temperature overnight (about 18 hours). Next morning the wells are washed with ELISA wash buffer [phosphate buffered saline (PBS), pH 7.4, 0.05% Tween®-20] and blocked with 3% BSA in PBS for 1 hour (75 µL/well). ELISA strips are stored, dry, at -20°C until needed.

To determine the antigenicity of particles, antisera are diluted using 1% BSA in PBS and 50 µL/well added to antigen-coated ELISA wells. Sera are incubated for 1 hour, washed with ELISA wash buffer and probed using an anti-mouse(IgG)-HRP (The Binding Site, San Diego, CA; HRP = horseradish peroxidase) conjugate (50 µL/well) or other appropriate antibody for 30 minutes. After washing with ELISA wash buffer the reaction is visualized by the addition of TM blue substrate (50 µL/well). After 10 minutes, the reaction is stopped by the addition of 1N H₂SO₄ (100 µL/well) and is read on an ELISA plate reader set at 450 nm.

2. Synthetic Peptide ELISA

A 20 amino acid residue synthetic peptide (NANP)₅ is diluted to a concentration of 2 µg/mL in coating buffer (50 mM sodium bicarbonate, pH 9.6) and coated onto the wells of ELISA strips (50 µL/well). Peptides are dried onto the wells by incubating overnight (about 18 hours), in a hood with the exhaust on. Next morning, the wells are washed with ELISA wash buffer (phosphate buffered saline, pH 7.4, 0.05% Tween®-20) and blocked with 3% BSA in PBS (75 µL/well) for 1 hour. ELISA strips are stored, dry, at -20°C until needed.

To determine antibody antigenicity of particles, antisera (monoclonal or polyclonal) are diluted using 1% BSA in PBS, and 50 µL/well are added to antigen-coated ELISA wells. Sera are incubated for 1 hour, washed with ELISA wash buffer, and probed using an anti-mouse(IgG)-HRP conjugate (as above at 50 µL/well) or other appropriate antibody for 30 minutes, washed again with ELISA wash buffer, and then visualized by the addition of TM blue substrate (50 µL/well). After 10 minutes, the reaction is stopped by the addition of 1N H₂SO₄ (100 µL/well) and read on an ELISA plate reader set at 450 nm.

B. Immunogenicity of Particles

To assay the immunogenicity of particles, mice are immunized, IP, with 20 µg of particles in Freund's complete adjuvant, and then boosted at 4 weeks with 10 µg in Freund's incomplete adjuvant. Mice are bled at 2, 4, 6, and 8 weeks.

C. Thermal Stability Protocol

Purified particles are diluted to a concentration of 1 mg/mL using 50 mM NaPO₄, pH 6.8 and sodium azide is added to a final concentration of 0.02% to prevent bacterial growth. Particles are incubated at 37° C and aliquots are taken at a desired time point. Samples are mixed with SDS-PAGE sample buffer (reducing) and run on 15% SDS-PAGE gels. Gels are stained using Coomassie Blue, and then analyzed.

D: Analytical Gel Filtration

Analysis of Hybrid particles

Analytical gel filtration analysis of purified hybrid HBC particles is performed using a 25 mL Superose[®] 6 HR 10/30 chromatographic column (Amersham Pharmacia # 17-0537-01) and a BioCAD[™] SPRINT Perfusion Chromatography System. The UV detector is set to monitor both wavelengths of 260 and 280 nm. The column is equilibrated with 3 column volumes (CV; about 75 mL) of buffer (50 mM NaPO₄, pH 6.8) at a flow rate of 0.75 mL/minute.

The particles to be analyzed are diluted to a concentration of 1 mg/mL using 50 mM NaPO₄, pH 6.8. 200 Microliters (μL) of the sample are then loaded onto a 200 μL loop and injected onto the column. The sample is eluted from the column with 50 mM NaPO₄, pH 6.8 at a flow rate of 0.75 mL/minute. Integration of the 280 nm trace was carried out using BioCAD[™] software (PerSeptive[™]) to provide the results.

Example 3: Determination of 280/260 Absorbance Ratios

Protein samples are diluted to a concentration of between 0.1 and 0.3 mg/mL using

phosphate buffered saline (PBS), pH 7.4. The spectrophotometer is blanked, using PBS, and the absorbance of the protein sample is measured at wavelengths of 260 nm and 280 nm. The absorbance value determined for a sample at 280 nm is then divided by the absorbance value determined for the same sample at 260 nm to achieve the 280/260 absorbance ratio for a given sample. The ratios were obtained for several samples, including native particles (HBc183), HBc particles truncated after residue position 149 (HBc149), and several HBc chimers that are identified elsewhere herein, are shown below in Table 8. Full length particles ICC-1559 are a preparation of the particles first reported in Neirynck et al., (Oct 1999) *Nature Med.*, 5(10):1157-1163, whereas full length particles ICC-1607 are similar particles in which the M2 polypeptide cysteines at polypeptide positions 17 and 19, (X₁₇ and X₁₉ of SEQ ID NO:9) were mutated to serine residues.

Table 8

Particle Number	Full Length, (F) or C-Terminal Truncated, (T)	280/260 Absorbance Ratio
HBc183	F	0.84
HBc149	T	1.59
1438	T	1.57
1473	T	1.64

1475	T	1.04
1492	T	1.33
1559	F	0.68
1560	T	1.36
1590	T	1.51
1603	T	1.68
1604	T	1.40
1605	T	1.26
1607	F	0.73

Example 4: Cysteine at the C-terminus
of Truncated HBc Particle

A. Addition of a Cysteine Residue
to the C-terminus of Hybrid HBc Particles

Using the polymerase chain reaction (PCR), genes expressing hybrid HBc particles can be easily mutated to introduce a cysteine or cysteine-containing peptide to the C-terminus of an HBc chimera that contains an added cysteine at the N-terminus. For example, a PCR oligonucleotide primer that encodes SEQ ID NO:256 can be used, in concert with a suitable second primer, to amplify a hybrid HBc gene and incorporate a cysteine codon between codon V149 and the stop codon. An exemplary construct is that referred to as ICC-1492 that is discussed hereinafter. See also, the preparation of V2.Pf1[N-M2(17-24/C19S)] that is discussed hereinafter.

Hepatitis B core particles can be truncated from 183 (or 185, depending on viral subtype) to 140 and retain the ability to assemble into particulate virus-like particles. Many groups have used particles truncated to amino acid 149 because amino

acid 150 represents the first arginine residue of the arginine-rich C-terminal domain.

Example 5: Influenza M2 Constructs

Recently, Neirynck et al., (Oct 1999) *Nature Med.*, 5(10):1157-1163 and WO 99/07839 reported the fusion of the 24 amino acid extracellular domain of M2 to the N-terminus of full-length HBc particles (HBc183), lacking amino acid residues 1-4. A schematic representation of that construct referred to herein as IM2HBc is shown below in which the 24-mer is linked to the N-terminus of HBc.

IM2HBc

MSLLTEVETPIRNEWGCRCNDSSD-HBc (5-183)

SEQ ID NO: 256

In one illustrative preparation, the M2 epitope was inserted into the immunodominant loop of hepatitis B core and particles referred to as ICC-1475 were successfully expressed and purified using techniques discussed previously for such insertions and purifications. A mutated version of the M2 epitope, in which two cysteine residues at M2 native positions 17 and 19 were substituted by alanine residues, was also expressed in the immunodominant loop (ICC-1473 particles) and the resulting particles purified. These two particles are illustrated schematically below.

ICC-1475

HBc (1-78) -GI-SLLTEVETPIRNEWGCRCNDSSD-EL-HBc (79-149)

SEQ ID NO: 257

ICC-1473

HBc (1-78) -GI-SLLTEVETPIRNEWGARANDSSD-EL-HBc (79-149) -C

SEQ ID NO: 258

The ICC-1473 particle construct yielded approximately 7-fold more purified particles when compared with the native sequence (ICC-1475). It remains to be determined if the mutation of the cysteine residues alters protective potential of the particles. However, epitopes delivered on the immunodominant loops of HBc are usually significantly more immunogenic as compared to when they are fused to other regions (including the N-terminus), and resulting particles exhibit reduced anti-HBc immunogenicity.

Particles have also been prepared in which the M2 N-terminal 24-mer epitope was fused to the N-terminus of C-terminal truncated hepatitis B core particles. That construct (ICC-1438) also contained the N-terminal pre-core sequence (SEQ ID NO:259). A similar construct was prepared that contained a single cysteine residue at the end of the hybrid protein (ICC-1492), in this case immediately after Val-149 of the HBc gene. These constructs are shown schematically below.

ICC-1438

MGISLLTEVETPIRNEWGCRCNDSSDELLGWLWGI-HBc(2-149)

SEQ ID NO:259

ICC-1492

MGISLLTEVETPIRNEWGCRCNDSSDELLGWLWGI-HBc(2-149)-C

SEQ ID NO:260

It should be noted that to guard against translation initiation from the natural HBc initiator methionine, the codon for that residue was mutated to code for an isoleucine residue. Residues contributed by EcoRI (GI) and SacI (EL) restriction sites are underlined. The pre-core sequence is recited between the underlined EL residues and "-HBc(2-149)".

Analysis by SDS-PAGE as discussed elsewhere herein, showed that upon preparation, the ICC-1438 monomer construct was unstable (Lane 2) as compared to the ICC-1492 (Lane 3), with HBc-149 (Lane 1), ICC-1475' (Lane 4) and ICC-1473 (Lane 5) serving as additional molecular weight controls on the SDS-PAGE gel in Fig. 10. The instability of the ICC-1438 monomers was not evident using analytical gel filtration of particles.

Both ICC-1475 (Fig.10, lane 4) and ICC-1473 (Fig.10, lane 5) were expected to have slightly lower molecular weights than ICC-1438 and ICC-1492, because the former two contain the M2 epitope inserted directly into the immunodominant loop and therefore lack the pre-core sequence (SEQ ID NO:259) present in ICC-1438 and ICC-1498. As expected, ICC-1492 was larger than ICC-1475 and ICC-1473; however, ICC-1438, which is identical to ICC-1492 save the C-terminal

cysteine residue, is clearly not larger than ICC-1475 and ICC-1473 due to an apparent cleavage.

A construct containing a M2 N-terminal extracellular sequence as discussed before linked to the HBc N-terminus (Domain I) or loop (Domain II) and also containing a cysteine residue at the C-terminus (Domain IV) of HBc is also contemplated.

To modify the amino-terminus of hybrid HBc particles containing immunodominant loop fusions to incorporate a cysteine residue, and minimal M2-derived sequence, a series of synthetic oligonucleotides are synthesized. To make V2.Pf1(N-M2(17-24/C17S)), the oligonucleotides M2(17-24/C17S)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 546 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which has been cleaved with the same two enzymes.

To make V2.Pf1[N-M2(17-24/C19S)], the oligonucleotides M2(17-24/C19S)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene in vector V2.Pf1. The resultant 540 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-223-3N, which had been cleaved with the same two enzymes.

M2 (17-24/C17S) -NcoI-F

M G S R C N D S S D I D P Y K E
 .GGCGCCATGGGGTCTAGATGTAACGATTCAAGTGACATCGACCCTTATAAAGA

F G

SEQ ID NO: 261

ATTTCG

SEQ ID NO: 262

M2 (17-24/C19S) -NcoI-F

M G C N D S S D I D P Y K E F G

SEQ ID NO: 263

GCGCCATGGGGTGTAAACGATTCAAGTGACATCGACCCTTATAAAGAATTTGG

SEQ ID NO: 264

Example 6: HBc Chimer Molecules With and Without
Both N- and C-Terminal Cysteine Residues

A series of HBc chimer molecule-containing particles was prepared that contained residues 1-24 of the influenza A, M2 protein peptide-bonded at or near the N-terminus of HBc whose C-terminus was truncated at residue 149. The component chimeric protein molecules contained different N-terminal sequences that included an M2 sequence or variant, and some contained a C-terminal cysteine residue.

All purified particles listed in Table 9, hereinafter, were analyzed by analytical size exclusion chromatography to assess the retention of particulate structure following purification. Particles designated ICC-1603, which contain no N-terminal cysteine residues, displayed evidence of disassembly back to sub-particulate structures (Fig. 3) because the protein eluted in the 1500 second

range (particles elute at approximately 1000 seconds).

Similar analysis of particles ICC-1590, which are similar to ICC-1603 ICC-particles except for the mutation of two serine residues to cysteine residues in the N-terminal M2 sequence, revealed that that construct remained particulate following purification, with elution occurring at around 1000 seconds, which is typical for a hybrid particle (Fig. 4). There was no evidence of disassembly for ICC-1590 particles.

Analysis of ICC-1560 particles, whose chimer protein also has two N-terminal cysteine residues, revealed that it too was particulate following purification, although it did exhibit some degree of disassembly (Fig. 5), suggesting that the stabilization was not quite as robust as it was for ICC-1590 particles. Comparison of the N-terminal configurations of ICC-1590 and ICC-1560 particles (Table 11, hereinafter), shows that the relative position of the two cysteine residues in ICC-1560 particles is shifted by 3 amino acid residues relative to ICC-1590 particles via the deletion of three amino acid residues (DEL), indicating that the cysteine residues may be required to be a minimal distance from the start of the core gene to enable optimal cross-linking.

Example 7: Particles With an M2 or M2 Variant

Sequence and A C-Terminal Cysteine Residue

ICC-1603 particles were shown in Fig. 3 to rapidly disassemble following purification. The HBc chimer molecules that comprise ICC-1605 particles are

similar to those of ICC-1603 particles, except that the ICC-1605 component chimer molecules have a single C-terminal stabilizing cysteine. A plasmid was made to direct the expression of ICC-1605 particles to investigate if the addition of a C-terminal cysteine residue to ICC-1603 particles could impart greater stability on the particle. Following purification, ICC-1605 particles were analyzed using analytical size exclusion chromatography (Fig. 6).

The results of this study demonstrated that particle stabilization was more complete than for the ICC-1603 particles, but incomplete compared to ICC-1590 particles, which contains two amino-terminal cysteine residues and no C-terminal stabilizing cysteine. Although a significant amount of ICC-1605 remained particulate, there was evidence of a heterogeneous mixture of sub-particulate structures that eluted over a broad range. These observations suggest that for this hybrid particle (ICC-1603), C-terminal stabilization as found in ICC-1605 particles was less complete than for the N-terminal stabilization found in ICC-1590 particles.

To investigate the compatibility of combined amino and carboxyl-terminal cysteine stabilization of hybrid particles, an expression plasmid was constructed to direct the expression of ICC-1604 particles. The component chimer molecules of ICC-1604 particles contain both the two amino-terminal stabilizing cysteine residues present in a native M2 polypeptide sequence (as in ICC-1590) as well as a C-terminal stabilizing cysteine (as in ICC-1605 particles). Analysis of ICC-1604 particles showed that they retained a homogeneous particulate state following purification (Fig. 7), indicating that the

$$\frac{d}{dt} \left(\frac{\partial L}{\partial \dot{x}} \right) = \frac{\partial L}{\partial x}, \quad \frac{d}{dt} \left(\frac{\partial L}{\partial \dot{y}} \right) = \frac{\partial L}{\partial y}$$

These particles differed only in the fact that the ICC-1438 component chimer molecule terminated at position 149 of HBc, whereas the ICC-1492 component chimer molecule terminated at 149 of HBc and contained a terminal cysteine at position 150 relative to the HBc of SEQ ID NO:1. When analyzed by analytical gel filtration, using an alternative but similar method to that discussed before, whereby particles elute at approximately 10 minutes, both constructs were shown to be particulate following purification (ICC-1438 in Fig. 8 and ICC-1492 in Fig.10). This study demonstrated the compatibility of amino- and carboxyl-terminal cysteine stabilization of truncated particles, and the tolerance of substantial variability in the amino acid sequence and distance between the N-terminal cysteine residues and start of the HBc gene.

Table 9

Construct Name	N-terminal Fusion	HBc N-term Start	Residues Between M2 and HBc	C-term End	Bound Nucleic Acid	C-term Cysteine Stab
ICC-1560	M2 (1-24)	D4	None	149	No	No
ICC-1603	M2 (1-24) (2C>2S)	D4	EL	149	No	No
ICC-1590	M2 (1-24)	D4	EL	149	No	No
ICC-1604	M2 (1-24)	D4	EL	149	No	Yes (C150)
ICC-1605	M2 (1-24) (2C>2S)	D4	EL	149	No	Yes (C150)
ICC-1438	M2 (1-24)	D2	ELLGWLWGI	149	No	No
ICC-1492	M2 (1-24)	D2	ELLGWLWGI	149	No	Yes (C150)

Table 10, below, shows an alignment that illustrates the configuration of the N-termini of HBeAg, and particles designated ICC-1590, ICC-1560, ICC-1603, ICC-1604 and ICC-1605. Sequences are aligned according to amino acid residue position 4 from the N-terminus of HBc of SEQ ID NO:1 that is shared by all constructs. N-terminal cysteine residues, when present, are underlined.

Table 10

<u>Construct Name</u>	<u>Sequence</u>	<u>SEQ ID NO</u>
HBeAg	SKL <u>C</u> LGWLWGMDID	266
ICC-1590/ICC-1604	MSLLTEVETPIRNEWG <u>C</u> <u>R</u> CNDSSDELD	267
ICC-1560		

MSLLTEVETPIRNEWGCRCNDSSD

268

ICC-1603/ICC-1605

MSLLTEVETPIRNEWGSRSDSSDELD

269

ICC-1438/ICC-1492

MGISLLTEVETPIRNEWGCRCNDSSDELLGWLWGIDID

270

Table 11, below, provides a tabulation of the results in which stability was assessed for particles containing an N-terminal influenza A M2 sequence or variant contemplated herein. As is seen, stable particles have been prepared from HBc chimer molecules that contain an N-terminal cysteine residue at a position of minus 14 (-14) relative to the N-terminus of the HBc sequence of SEQ ID NO:1 to about the N-terminus itself.

Table 11

Construct Name	Amino Acids Between HBc D4 and N-terminal Cysteine Residues		C-terminal Cysteine Stabilization	Stable Particle Formed
	Cys 1	Cys 2		
HBeAg	-	9	No	No
ICC-1603	-	-	No	No
ICC-1605	-	-	Yes	Yes/No
ICC-1590	9	7	No	Yes
ICC-1604	9	7	Yes	Yes
ICC-1560	6	4	No	Yes
ICC-1438	18	16	No	Yes
ICC-1492	18	16	Yes	Yes

Each of the patents and articles cited herein is incorporated by reference. The use of the article "a" or "an" is intended to include one or more.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.